EFFECT OF GENETIC ALTERATION OF CYTOSINE ARABINOSIDE METABOLIZING ENZYMES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

CHUMPHORN BANKLAUI

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(CLINICAL PATHOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2010**

COPYRIGHT OF MAHIDOL UNIVERSITY

Thesis
entitled

EFFECT OF GENETIC ALTERATION OF CYTOSINE ARABINOSIDE METABOLIZING ENZYMES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

…………………………………………..
Miss Chumphorn Banklaui
Candidate

...………………………………………..
Assoc.Prof. Suntaree Apibal,
M.Sc.
Major-advisor

…………………………………………..
Assoc.Prof. Samart Pakakasama,
M.D.
Co-advisor

…………………………………………..
Lect. Sumalee jindadamrongwech,
Ph.D.
Co-advisor

……………………………………….
Prof.Bunchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

…………………………………………..
Assist.Prof. Pitak Santanirand,
Ph.D.
Program Director
Master of Science Programme
in Clinical Pathology
Faculty of Medicine
Ramathibodi Hospital
Mahidol University

Thesis
entitled

EFFECT OF GENETIC ALTERATION OF CYTOSINE ARABINOSIDE METABOLIZING ENZYMES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Clinical Pathology)
on
January 4, 2010

…………………………………………..
Miss Chumphorn Banklaui
Candidate

...………………………………………...
Assoc.Prof.Surangrat Srisurapanon,
Ph.D.
Chair

………………………………………….
Assoc.Prof.Suntaree Apibal,
M.Sc.
Member

…………………………………………..
Lect. Sumalee Jindadamrongwech,
Ph.D.
Member

………………………………………….
Assosc.Prof. Samart Pakakasama,
M.D.
Member

……………………………………….
Prof. Banchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

…………………………………………..
Prof. Rajata Rajatanavin,
M.D., F.A.C.E.
Dean
Faculty of Medicine
Ramathibodi Hospital,
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my honest gratitude and deepest appreciation to the my adviser, Associate Professor Suntaree Apibal, Associate Professor Samart Pakakasama and Dr. Sumalee Jindadamrongwech for their guidance, valuable advice, supervision and encouragement throughout the course of this thesis. They were never lacking in kindness and support.

I also would like to sincerely thank Associate Professor Surangrat Srisurapanon for serving as my chair and external examiner of the thesis defense.

My special thanks are directed to Mr.Ruchchadol Sawangpanich, staff of laboratory at the division of Pediatric Hematology-Oncology Unit, Department of Pediatric, Ramathibodi Hospital for their useful technical guidance and support. Furthermore, my appreciativeness is also to all staff of medical genetic Unit, Department of Medicine , Ramathibodi Hospital and Miss Wansa Banyatsupasin, Miss Kobkorn Pongjantharsatien, Miss Noppawan Tangbubpha and all staff of Blood disease diagnosis center, Department of Pathology. for their great help for equipment in this thesis. I am grateful for Assistant professor Sumalee Singhaniyom, Public health Faculty, Mahidol University, Miss Teerarat Tassanapitikul, statistician of Ambulatory unit, Department of Pediatric and Miss Umaporn Udomsabpayakul, statistician of Research center, Ramathibodi hospital.

I wish to thank all friends and all member of the Master Degree Programme in Clinical Pathology for helpful, encouragement and warm atmosphere.

Most of all, I am very grateful to my lovely family for their love, entirely care, and encouragement. This would never have been possible without all of you.

Chumphorn Banklaui

EFFECT OF GENETIC ALTERATION OF CYTOSINE ARABINOSIDE METABOLIZING ENZYMES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

CHUMPHORN BANKLAUI RACP/M 5036436

M.Sc. (CLINICAL PATHOLOGY)

THESIS ADVISORY COMMITTEE: SUNTAREE APIBAL, M.Sc., SAMART PAKAKASAMA, M.D., SUMALEE JINDADAMRONGWECH Ph.D.

ABSTRACT

The resistance to cytosine arabinoside (AraC) chemotherapeutic agents is the major problem in the treatment of acute leukemia. The objective of this thesis was to evaluate the effect of genetic alteration of two important genes encoded in the AraC metabolizing enzyme; the deoxy cytidine kinase (dCK) gene with two single nucleotide polymorphisms (SNPs) -360 C>G, -201 C>T and the cytidine deaminase (CDA) gene with two SNPs; 79 A>C, 208 G>A. They were evaluated for genotypes using the RFLP and ARMS PCR techniques and for mRNA expression using the quantitative Real time PCR technique.

We performed 94 peripheral blood tests on children with acute lymphoblastic leukemia (ALL) and 100 blood tests on a normal control group from the general population. All genes have 3 possible genotypes; either wild, heterozygous, or variant. The dCK (-360 C>G,-201 C>T) gene in the ALL test group was composed of 72%, 27% and 1% respectively, while the normal control group was composed of 70%,26% and 4% , respectively. The CDA gene, 79 A>C in the ALL test group was composed of 87%,13% and 0%, respectively while the normal control group was composed of 81%,18% and 1%, respectively. The CDA 208 G>A had only one genotype which was 100% wild type. The mRNA expression was studied using a quantitative Real time PCR technique on 44 RNA extracted from bone marrow of recently diagnosed ALL subjects, and calculated using the $2^{(-\Delta CT)}$ formula.

We found that there was an association between genotypes and toxicity in ALL patients with heterozygous and variant genotypes (-360 CG/-201 CT,-360 GG/-201 TT) in that there was an AraC related mucositis occurring. However, there was no significant association of AraC toxicity in the alleic genotype of CDA, but this gene was connected to the improvement of patients. It showed that there was an association between Mininmal Residual disease (MRD) and mRNA expression. Neither the genes nor the genotypes were significantly associated with mRNA expression. In conclusion, the results of this study indicated that dCK and CDA were important genes in AraC metabolization.

KEY WORDS: ACUTE LYMPHOBLASTIC LEUKEMIA / RFLP-PCR / ARMS-PCR /QUANTITATIVE REAL TIME PCR / mRNA EXPRESSION

104 pages

ผลกระทบของความเปลี่ยนแปลงของยีนที่เกี่ยวข้องกับเมตาบอลิซึมของยา Cytosine arabinoside ในเด็กโรคมะเร็งเม็ดเลือดขาวเฉียบพลันลิมโฟบลาสต์

EFFECT OF GENETIC ALTERATION OF CYTOSINE ARABINOSIDE METABOLIZING ENZYMES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

ชุมพร บ้านกล้วย 5036436 RACP/M

วท.ม. (พยาธิวิทยาคลินิก)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : สุนทรี อภิบาล, M.Sc., สามารถ ภคกษมา, M.D.,
สุมาลี จินดาดำรงเวช, Ph.D.

บทคัดย่อ

AraC เป็นยาตัวหนึ่งที่ใช้ในการรักษาโรคมะเร็งชนิดเฉียบพลัน และปัญหาการดื้อยาของ AraC นั้นก็เป็นปัญหาที่สำคัญต่อการรักษาด้วย การศึกษาครั้งนี้เป็นการประเมินผลกระทบของการเปลี่ยนแปลงของยีน dCK และ CDA ซึ่งเกี่ยวข้องกับการเมตาบอไลต์ยา AraC ในตำแหน่งพอลิมอฟิซึมที่ -360 C>G, -201 C>T และ 79 A>C, 208 G>A ของยีน dCK และ CDA ตามลำดับ โดยทำการศึกษา genotype และ mRNA expression ของยีน

เทคนิค RFLPและ ARMS PCR เป็นวิธีที่นำมาใช้ในการศึกษา genotype โดยทำการวิเคราะห์ตัวอย่างของคนไข้เด็กมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันลิมโฟบลาสต์ จำนวน 94 ตัวอย่าง และตัวอย่างของกลุ่มคนปกติจำนวน 100 ตัวอย่าง ผล genotype ของแต่ละยีนประกอบด้วย wild type, heterozygous และ variant ซึ่งความถี่ในคนไข้เด็กมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันลิมโฟบลาสต์และกลุ่มคนปกติ ในยีน dCK เป็น 72%,27%,1% และ 70%,26%,4%ตามลำดับ, ยีน CDA 79 A>C เป็น 87%, 13%,0% และ 81%,18%,1% ตามลำดับ ส่วนยีน CDA 208 G>A นั้นพบเพียงgenotype ชนิด wild typeเพียงชนิดเดียวส่วนการศึกษา mRNA expression นั้นศึกษาโดยใช้เทคนิค quantitative Real time PCR ในตัวอย่าง RNA ที่ได้จาก bone marrow ของคนไข้เด็กมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันลิมโฟบลาสต์ ซึ่งคำณวนโดยใช้สูตร$2^{(-\Delta CT)}$.

การศึกษาความสัมพันธ์ระหว่าง genotype กับการเกิดพิษของยา เราพบว่าในคนไข้มะเร็งเม็ดเลือดขาวชนิดเฉียบพลันลิมโฟบลาสต์ที่มี genotype ชนิด heterozygous และ variant ของยีน dCK (-360 C>G,-201 C>T) นั้นจะส่งผลให้เกิดอาการ mucositis ต่อคนไข้ได้มากกว่าคนไข้ที่มี genotype ชนิดอื่นๆส่วนยีน CDA นั้นไม่พบความสัมพันธ์กับการเกิดพิษของยาแต่พบว่ายีน CDA นั้นสัมพันธ์กับผลการรักษาคนไข้ โดยพบความสัมพันธ์ระหว่าง MRD และ mRNA expression ส่วนการศึกษา mRNA expressionในแต่ละ genotype ของยีนไม่พบความแตกต่างกัน โดยสรุปพบว่ายีน dCK และ CDA นั้นเป็นยีนที่สำคัญในการเมตาบอไลท์ยา AraC

104 หน้า

CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
ABSTRACT (ENGLISH)	iv
ABSTRACT (THAI)	v
LIST OF TABLES	viii
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xi
CHAPTER I INTRODUCTION	1
CHAPTER II OBJECTIVE	4
CHAPTER III LITERATURE REVIEW	5
3.1 Diagnosis and Classification of acute leukemia	5
3.1.2 Immunophenotypic Classification	7
3.2 Clinical Characteristics	9
3.3 Laboratory Features	9
3.4 Treatment	9
3.4.1 Remission induction	10
3.4.2 Intensification (Consolidation)	10
3.4.3 Continuation treatment	11
3.5 Cytarabine (Cytosine arabinoside ,AraC)	11
3.5.1 AraC mediated Cytotoxicity	13
3.5.1.1 Inhibition of DNA polymerase and DNA incorporation	13
3.5.1.2 AraC accumulation and retention	13
3.5.2 Pharmacokinetic of AraC	13
3.5.3 Side effect of AraC	14
3.5.4 dCK gene	14
3.5.5 CDA gene	14

CONTENTS (cont.)

Page

3.6 Genotyping Method 15

3.6.1 Polymerase chain reaction -Restriction Fragment length polymorphism (PCR-RFLP) Analysis 15

3.6.2 Amplification Refractory Mutation System (ARMS) 16

3.6.3 Direct DNA Sequencing 17

3.7 Quantitative real-time PCR (RQ-PCR) 18

CHAPTER IV MATERIALS AND METHODS 22

4.1 Biological samples 22

4.2 Materials 22

4.3 Methods 29

CHAPTER V RESULT 37

CHAPTER VI DISCUSSION 84

CHAPTER VII CONCLUSION 89

REFERENCES 90

APPENDIX 102

BIOGRAPHY 104

LIST OF TABLES

Table		Page
3.1	Lymphoblastic leukemia	6
3.2	Immunophenotype in Acute Lymphoblastic Leukemia	8
4.1	Primers for PCR	24
4.2	Primers for ARMS	25
4.3	Restriction enzymes for PCR-RFLP	27
4.4	Primers for Real-time PCR	29
5.1	Genotyping of ALL patients	49
5.2	Genotyping of Control	54
5.3	Frequency of genotype and alleles of dCK gene (-360 C>G,-201 C>T)	59
5.4	Frequency of genotype and alleles of CDA gene (208 G>A)	59
5.5	Frequency of genotype and alleles of CDA gene (79 A>C)	59
5.6	Result of association between genotype of dCK -360 C>G,-201 C>T with AraC toxicity (low dose)	61
5.7	Result of association between genotype of dCK -360 C>G, -201 C>T with AraC toxicity (High dose)	62
5.8	Result of association between allele of dCK -360 C>G,-201 C>T with AraC toxicity (low dose)	63
5.9	Result of association between allele of dCK -360 C>G,-201 C>T with AraC toxicity (High dose)	64
5.10	Result of association between genotype of CDA (79 A>C) with AraC toxicity (low dose)	65
5.11	Result of association between genotype of CDA (79 A>C) with AraC toxicity (High dose)	66
5.12	Result of association between allele of CDA (79 A>C) with AraC toxicity (low dose)	67
5.13	Result of association between allele of CDA (79 A>C) with AraC toxicity (High dose)	68

LIST OF TABLES (cont.)

Table		Page
5.14	Result of association between Genotype and MRD	69
5.15	Result of association between Genotype and subtype of ALL in low dose phase	71
5.16	Result of association between Genotype and subtype of ALL in high dose phase	72
5.17	Result of association between Genotype with B-lineage and T-lineage in low dose phase	73
5.18	Result of association between Genotype with B-lineage and T-lineage in high dose phase	74
5.19	Result of association between Genotype with early pre B cell and Pre B cell in low dose phase	75
5.20	Result of association between Genotype with early pre B cell and Pre B cell in high dose phase	76
5.21	The result of mRNA expression level of dCK and CDA normalized with beta actin	80
5.22	The relative mRNA level of dCK and CDA expression	82
5.23	The association between mRNA expression and MRD	83

LIST OF FIGURES

Figure		Page
1.1	AraC metabolism within cells	1
3.1	AraC metabolism within cells	12
3.2	Reaction for RFLP technique	16
3.3	Principle for SNPs genotyping by ARMS	17
3.4	Reaction of DNA sequencing	18
3.5	Fluorescence for Real time PCR	19-20
5.1	The amplification of dCK gene	37
5.2	The amplification of CDA 208 G>A	38
5.3	The restriction fragment of dCK-360 C>G	39
5.4	The restriction fragment of dCK-201 C>T	40
5.5	The restriction fragment of CDA 208 G>A	41
5.6	The amplification of ARMS PCR	42
5.7	Chromatogram of direct sequencing for dCK-360 C>G wild type (CC)	43
5.8	Chromatogram of direct sequencing for dCK-360 C>G, Heterozygous (CG)	43
5.9	Chromatogram of direct sequencing for dCK -360 C>G, Variant (GG)	44
5.10	Chromatogram of direct sequencing for dCK -201 C>T wild type (CC)	44
5.11	Chromatogram of direct sequencing for dCK -201 C>T, Heterozygous (CT)	45
5.12	Chromatogram of direct sequencing for dCK -201 C>T, Variant (TT)	45
5.13	Chromatogram of direct sequencing for CDA 208 G>A, wild type (GG)	46
5.14	Chromatogram of direct sequencing for CDA 79 A>C, wild type (AA)	46
5.15	Chromatogram of direct sequencing for CDA 79 A>C, Heterozygous	47
5.16	Chromatogram of direct sequencing for CDA 79 A>C, Variant	47

LIST OF ABBREVIATIONS

ACTB	=	Actin, Beta
Ala	=	Alanine
ALL	=	Acute lymphoblastic leukemia
AML	=	Acute myeloid leukemia
ANC	=	Absolute nuetrophil count
AraC	=	Cytosine arabinoside, Cytarabine
AraCMP	=	Arabiniside monophosphate
AraCTP	=	Arabinoside triphosphate
ARMS	=	Amplification refractory mutation system
AraU	=	Uracil arabinoside
bp	=	Base pair
°C	=	Degree of celcius
CD	=	Cluster designation
CDA	=	Cytidine deaminase
CNS	=	Central nerverse system
Ct	=	Threshold cycle
DIC	=	Disseminated intravascular coagulopathy
dCMPD	=	Deoxycytidylate deaminase
dCTP	=	Deoxycytidine triphosphate
dATP	=	2’deoxyadenoside 5’triphosphate
dCTP	=	2’deoxycytidine 5’ triphosphate
dCyd	=	deoxycytidine
dGTP	=	2’deoxyguanosine 5’triphosphate
dNTP	=	Deoxynucleotide triphosphate
dTTP	=	2’deoxytymidine 5’triphosphate
dCK	=	Deoxycytidine kinase
DNA	=	Deoxyribonucleic acid

LIST OF ABBREVIATIONS (cont.)

dsDNA	=	Double strand deoxyribonucleic acid
EDTA	=	Ethylene diamine tetracetate
FAB	=	French-American-British
FRET	=	Forster resonance energy transfer
g	=	Gram
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
Gln	=	Glutamine
Hb	=	Hemoglobin
HCl	=	Hydrochloric acid
hENT1	=	Human equilibrative nucleoside transporters 1
HLA-DR	=	Human leukocyte antigen subclass DR
HScs	=	Hematopoietic stem cells
IgH	=	Immunoglobulin heavy chain
KCl	=	Potassium chloride
Lys	=	Lysine
m	=	Milli (10^{-3})
M	=	Molar
mg	=	milligram
$MgCl_2$	=	Magnesium chloride
min	=	Minute
MLL	=	Mix lineage leukemia
MRD	=	Minimal residual disease
mRNA	=	Messenger ribonucleic acid
PCR	=	Polymerase chain reaction
pH	=	Power of Hydrogen ion
Plt	=	Pletlet
PN I	=	Pyrimidine nucleotidase I

LIST OF ABBREVIATIONS (cont.)

RFLP	=	Restriction fragment length polymorphism
RNA	=	Ribonucleic acid
RQ	=	Quantitative real time
rSNP	=	Regulatory region single-nucleotide polymorphism
RT	=	Reverse transcription
sec	=	Second (s)
SNPs	=	Single-nucleotide polymorphism
TBE	=	Tris-borate-ethylene diamine tetraacetic acid
TdT	=	Terminal deoxynucleotide1 transferase
Thr	=	Threonine
Tm	=	Melting temperature
U	=	Unit (s)
UV	=	Ultraviolet
μ	=	Micro (10^{-6})
WBC	=	White blood cell
WHO	=	World Health Organization

CHAPTER I
INTRODUCTION

Background and the problem

Acute lymphoblastic leukemia (ALL) is the most common disease in childhood and young adulthood with a peak incidence at 2-5 years of age [1]. The antimetabolite cytosine arabinoside (AraC) is a deoxycytidine analog [2] which been used either alone or in combination with other chemotherapeutic agents for the treatment of acute myeloid leukemia, relapsed and refractory acute lymphoblastic leukemia and other malignancies [3]. The mechanism of Ara-C, a prodrug is intracellularly phosphorylated by deoxycytidine kinase (dCK) to 1-β-D-arabinofuranoxyl cytosine-5'-triphosphate (Ara-CTP)[4]. Its cytotoxicity is belived to result from a combination of DNA polymerase inhibition and from incorporation of Ara-CTP into DNA [5], incompetition with deoxycytidine triphosphate (dCTP). This incorporation causes chain termination and result in a block of DNA synthesis [3, 5] Figure 1.1 .

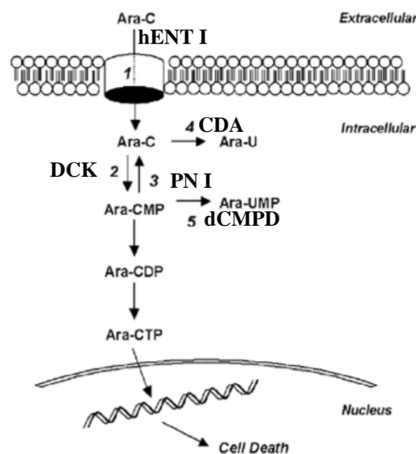


Figure 1.1: AraC metabolism within cells [2]

1. hENT1 (Human equilibrative nucleoside transporters 1)
2. dCK (Deoxy cytidine kinase)
3. PN I (Pyrimidine nucleotidase I)
4. CDA (Cytidine deaminase)
5. dCMPD (Deoxycytidylate deaminase)

Moreover, cytidine deaminase (CDA) is the other important gene involve in metabolizing enzyme for Ara-C. Up to the present knowledge, cytidine deaminase (CDA) and deoxy cytidine kinase (dCK) gene are the very important genes involved in metabolize enzyme for AraC. An alteration of CDA might have caused the severe drug toxicity as well as dCK and might be severed as genetic marker for predictive drug responsiveness

dCK gene

dCK has two single nucleotide polymorphisms (SNPs) in the regulatory region (rSNPs) with high allele frequencies. These two rSNPs (-201C>T and -360C>G) form two major haplotypes. Indicating that rSNP haplotypes of dCK may serve as a genetic marker for predicting drug responsiveness, which will be beneficial in establishing more effective AML chemotherapeutic regimens[7], however dCK is fairly conserved in Caucasians. There is still considerable interethnic variability of dCK polymorphisms, and this may partially explain erratic inter-patient and interethnic variability in response to nucleoside drugs. In particular, the higher allelic frequency of −360C>G/−201C>T in Asians relative to Caucasians might predispose Asians to nucleoside drug-associated toxicity [8]. The previous reported also revealed the decreased level of dCK mRNA expression or significant decreased in the dCK activity which also involved as one of the mechanisms responsible for clinical resistance to Ara-C [9-17] .

CDA gene

The studying about drug toxicity associated with single-nucleotide polymorphism of CDA gene by using two non-synonymous SNPs, 79A>C (Lys27Gln) and 208G>A (Ala70Thr) [18-22], revealed the ethnic or racial differences in the allele frequencies of these SNPs. The remarkable reduction in activity of 70Thr CDA was

reported in vitro [19] and in vivo [22], where as marginal reduction in activity of 27Gln CDA was observed in vitro [19, 21]. Fitzgerald *et al* (2006)[23] investigated SNPs in the promoter region of CDA in vitro and in vivo, and found that some promoter CDA haplotypes might affect CDA activity and homozygous 208G>A alteration in CDA might have caused the severe drug toxicity [24].

The study of CDA in several cell lines of evidence have showed the increased level of CDA in the development of resistance to Ara-C [25, 26] as well as the correlation of CDA mRNA with CDA enzyme activities in AML blasts which was the other mechanisms of resistanc to Ara-C [27] .

Ara-C is a drug component in the treatment protocol of childhood ALL at Faculty of Medicine Ramathibodi Hospital. The patients receive Ara-C during induction of remission and reinduction Π phase. Therefore, our study try to investigate the association between SNPs of Ara-C metabolizing enzyme (CDA, dCK) and toxicities during those periods. Remission status after the induction period will be included for assessment of those SNPs effects. The effect of CDA and dCK on their mRNA expressions also are explored. These data may explain the basic mechanism of their genetic alteration.

CHAPTER II
OBJECTIVE

1. To study the association between polymorphisms of AraC metabolizing enzymes and outcome of childhood with ALL
2. To evaluate the association between polymorphisms of AraC metabolizing enzymes and their expression
3. To study the frequencies of AraC metabolizing enzyme polymorphisms in Thai population

CHAPTER III
LITERATURE REVIEW

Acute leukemia is defined as the malignant accumulation of transformed hematopoietic progenitor cells affects both children and adults [1]. Leukemic blast cells retain the capability of self-renewal, but unlike normal hematopoietic stem cells (HSCs), they have limited or no potential for terminal differentiation. However, acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in children, representing nearly one third of all pediatric cancers, with peak prevalence between the ages of 2 and 5 years. Epidemiology of these malignancies have emphasized specific subgroups, as defined by morphology cytochemistry, immunological marker, cytogenetic features, or molecular markers. Among these techniques, the morphologic examination has remained the gold standard in establishing the diagnosis and guiding the selection of other techniques to classified ALL subgroups.

3.1 Diagnosis and Classification of acute leukemia

Accurate diagnosis and classification of acute leukemia is important for the selection of the appropriate therapy. According to the French-American-British (FAB) classification system for the diagnosis of acute leukemia, blasts must account for more than 30% of the nucleated cells in the peripheral blood or bone marrow aspirate. Acute myeologeneous leukemia (AML) and acute lymphoblastic leukemia (ALL) are distinguished according to the FAB classification system based on the morphologic features of the blasts. AML is divided into eight morphologic subtypes based on apparent level of differentiation: undifferentiated (M0), granulocytic (M1-M2), progranulocytic (M3), monoblastic (M4-M5), erythroblastic (M6), and megakaryoblastic (M7), and ALL is difined as three subtypes (L1,L2 and L3) according to (a) the occurrence of individual cytological features, and (b) the degree of heterogeneity in the distribution among the leukemic-cell population of some or all of

these features. The features considered are: cell size, nuclear chromatin, nuclear shape, nucleoli, amount and basophilic of cytoplasm [28].

For each of the features considered, up to 10% of the cells may depart from that characteristic of the type. A summary of the characteristics of each type is given in Table 3.1.

TABLE 3.1 . Lymphoblastic leukaemias

Cytological features	L 1	L2	L3
Cell size	Small cells predominate	Large, heterogeneous in size	Large and homogeneous
Nuclear Chromatin	Homogeneous in any one case	Variable- heterogeneous in any one case	Finely stippled and homogeneous
Nuclear shape	Regular, occasional clefting or indentation	Irregular; clefting and indentation common	Regular-oval to round
Nucleoli	Regular-oval to round	One or more present, often large	Prominent; one or more vesicular
Amount of cytoplasm	Scanty	Variable; often moderately abundant	Moderately abundant
Basophilia of cytoplasm	Slight or moderate, rarely intense	Variable; deep in some	Very deep
Cytoplasmic vacuolation	Variable	Variable	Often prominent

L1-type blast cells are predominantly small (up to twice the diameter of a small lymphocyte). Although characteristically the cells vary little in size, heterogeneity is compatible with L1 if all the other features are present. The nuclear chromatin is variable from case to case but homogeneous in any one case. It is usually finely dispersed but may appear clumped in the smaller cells. The nuclear shape is regular, but the presence of at least some degree of clefting, indentation, or folding is compatible with L1**.** Nucleoli are often not visible; the nucleolus when present is usually small and not prominent. The cytoplasm is usually scanty (high nuclear

cytoplasmic ratio). Deep cytoplasmic basophilia is uncommon. Characteristically, L1 is homogeneous in respect of all the features listed.

L2-type blast are larger than twice the size of small lymphocytes. The nuclear chromatin varies from finely dispersed to coarsely condensed, and is characteristically heterogeneous in any one case. Nuclear clefting, indentation and folding are characteristic and gross irregularities in shape are common. Nucleoli are nearly always present; they are variable in size and number often large. The amount of cytoplasm in the cells of any one case is variable and often abundant. Cytoplasmic basophilia is also variable and may be marked in some cases. Characteristically, L2 is heterogeneous in respect of all the features listed and there is consequently more variation between different cases than in L1 as well as in any one case .

L-3 type blast cell (Burkitt type), The cells are large and characteristically homogeneous. They have a dense but finely stippled nuclear chromatin. The nucleus is oval to round and regular. One or more prominent vesicular nucleoli are present in the majority of cells. The cytoplasm is moderately abundant and completely surrounds the nucleus. Intense cytoplasmic basophilia is present in every cell. L3 is characteristically homogeneous in respect of every feature listed, both from case to case, and in an individual case. A high mitotic index (about 5%) is characteristic. Prominent cytoplasmic vacuolation is often present in a majority of the cells; vacuolation may also be present in L1 and L2**,** but is usually less prominent. More recently, the world Health Organization suggested that the threshold for the diagnosis of acute leukemia is lowered to 20% blasts [29].

3.1.2 Immunophenotypic Classification

This is a more clinically relevant classification of ALL and is based on expression of certain antigens on the surface of leukemic cells. Expression of antigens on the cell surface indicates the specific step in differentiation where transformation occurred. Several classifications have been proposed for normal and leukemic lymphocytes.

Although this classification is useful clinically, one must be cautious of several factors. First, the phenotype of the lymphoblasts may not correlate with any normal phenotype, including some cases with simultaneous expression of antigens

normally present at different ends of the differentiation spectrum, although some of these lymphoblasts may actually have a rare normal counterpart.

The most common is CD10, also known as common ALL antigen, which is a membrane-bound neutral endopeptidase. It can be expressed in B- and T-cell leukemia CD34 is a marker of a very early pluripotential cell, including the stem and is most commonly expressed in non-T, non-B cell ALL cases. Co-expression of CD38 on CD34-positive cells is a marker for lineage commitment, is present on 20% of normal bone marrow cells as well as activated plasma cells and T cells, and is a common marker in T cell and B-cell leukemias. Another marker of activation, CD71, is more common in T-cell than in B-cell leukemias. The immunologic classification of ALL correlates with clinical characteristics with certain features associated with specific subtypes [30].

Table 3.2 Immunophenotype in Acute Lymphoblastic Leukemia: Expression of Differentiation Markers

Type	Surface marker
B –lineage	
Early pre B	HLA-DR+, TdT+, CD10+, CD19+, CD34+
Common ALL	HLA-DR+, TdT+, CD19+, CD10+
Pre B	HLA-DR+, TdT+, CD10+, CD19+, CD20+, CD34+, cIgH+,
Mature B cell ALL	HLA-DR+, CD19+, CD20+, sIgH+
T –lineage	
T cell ALL	CD2+, CD3+, CD4+, CD5+, CD7+, CD8+

CD: Cluster of differentiation, HLA-DR: Human leukocyte antigen subclass DR,TdT: Terminal deoxynucleotidyl tranferase,c:cytoplasmic, s:surface ,IgH: Immunoglobulin heavy chain

3.2 Clinical Characteristics

The signs and symptoms of ALL are variable. Most case have an acute onset, the presenting features generally reflex the degree of bone marrow failure [31].

Fever is the most common finding, occurring in approximately 50-60 % of patients. The patients are generally neutropenia and the neutrophil may be function abnormally. fatigue, anemia , bone pain and other symptoms can see in patients.

Patients with ALL have lymphadenopathy in up to 80% and hepatomegaly and/or splenomegaly in up to 75% of the cases. Other organs may also be involved, such as the kidney cortex (in one third of cases), heart, eyes, and gastrointestinal tract, Central nervous system (CNS).

3.3 Laboratory Features

Most of ALL patients at diagnosis (50-60%), the leukocyte count is greater than 10 X 10^9/L, only 10% of them have leukocyte count greater than 100 X10^9/L. Another 30-40% have leukocyte counts less than 10 X 10^9/L. More than 90% of patients present with platelet counts greater than 150 X 10^9/L and two thirds lesser than 50 X 10^9/L coagulopathies, including disseminated intravascular coagulopathy (DIC), may be seen in ALL at presentation or during therapy. Normocytic, normochromic anemia and reticulocytopenia are nearly universal. One third of patients have low levels of immunoglobulins, which may be a poor prognostic factor. The bone marrow is commonly hypercellular, rarely it is hypoplastic or aplastic. The most common way of determining the lymphoid origin of an acute leukemia is by histochemistry.

3.4 Treatment

The recently improved cure rate of ALL can be attributed mainly to the development of more effective chemotherapeutic regimens through successive well-designed clinical trials. In children, ALL is a highly curable disease, with cure rates ranging from 60% to 85%. Therapy for adults with ALL has followed that for children with ALL. About 75% of adults with ALL (range, 65-90%) achieve a complete remission, but despite significant progress in the last three decades, only 20-40% are

cured[30]. In patients with mature B-cell ALL, short term (2–8 months) regimens of intensive chemotherapy primarily based on cyclophosphamide, methotrexate, cytarabine, and intrathecal therapy, currently result in cure rates of 74–87% [32]. In several recent clinical trials, high-dose cytarabine, high-dose methotrexate, and intensive consolidation/ reinduction therapy seem to improve outcome[33, 34].

Treatment is ultimately the most important prognosis factor in ALL. Patients are difference features so they are classify into three groups; low risk group, standard risk group and high risk group. This classification base on difference feature, leukocyte count, MLL gene rearrangement, minimal residual disease (MRD), karyotype etc. Each type of risk group have difference regimen for treatment.

For all other patients, the basic approach to therapy consists of a brief remission-induction phase, followed by intensification (consolidation) therapy, and then long-term continuation treatment. All patients require treatment for sub clinical leukemia of the CNS, which should be initiated early in the form of intrathecal therapy.

3.4.1 Remission induction

The first goal of therapy is to induce complete remission with restoration of normal hemopoiesis. The induction regimen invariably includes a glucocorticoid (prednisone, prednisolone, or dexamethasone), vincristine, and at least one other agent (asparaginase or an anthracycline). The addition of another drug, cyclophosphamid, may improved outcome of T-ALL patients [35]. With improvement in supportive care and chemotherapy, the rate of complete remission is from 97% to 99% [1, 31, 36-42]. Only 1 % of the patients die from toxicity or failure from treatment during remission induction [31]. The patients who fail from this treatment have a short survival, or higher rate of relapse. The study of minimal residual disease (MRD) showed that the patients with 1% blast had poor outcome as induction failure.

3.4.2 Intensification (Consolidation)

Reducing the risk of relapse ALL, consolidation treatment is generally used to eracidate drug resistant residual leukemia cells. The consolidation has been valuable in the treatment of children with ALL. The important of this phase is no

longer disputed but lack of the best regimen and their duration. Commonly used regimens for childhood ALL include high-dose metrotrexate with mercaptopurine or with teniposide and cytarabine and asparaginase has significantly contributed to a cure rate of 70-80 % among children with ALL[30, 43]**.**The use of difference regimen for consolidation phase reveal the effective treatment for subtype of leukemia, example , using asparaginase or high dose of metrotrexate for this phase showed the improvement in T-lineage ALL in Dana-Farber Cancer institute Consortium and the children cancer group [31].

3.4.3 Continuation treatment

The ALL patients need continuation phase for the prevention of relapse. In early studies, short induction protocol and discontinuation after 15-65 weeks, were associated with high relapse rates. Patients with ALL have long time for this phase about 2–2.5 years, however, two-third of childhood ALL patients could be treated successfully within 1 years [44]. The mature B-cell ALL patients did not have this phase, usually receive 3-8 months of dose intensive consolidation therapy, the result of disease free survival rates of 40-60 %. The backbone of ALL continuation regimen are the combination of weekly metrotrexate and daily mercaptopurine. By virtue of its inhibiting effect on de novo purine synthesis, metrotrexate is synergistic with mercaptopurine as well as the other drug added in this phase, including duanorubicin, prednisone, doxorubicin, cyclophosphamide et al.

3.5 Cytarabine (Cytosine arabinoside ,AraC)

Cytosine arabinoside(1-β-D-arabinofuranosylcytosine, AraC) is deoxycytidine (dCyd) analogue, cytosine which was used for the treatment of acute leukemia and lymphoma. AraC is an important antimetabolite used in remission induction for patients with acute leukemia[45] as well as the most effective single agent of induction remission for acute myeloid leukemia (AML) and in many regimen for other acute leukemia including acute lymphoblastic leukemia (ALL) and non-Hodkin's lymphoma[46-48]. The activity of AraC depends on the conversion to its cytotoxic triphosphate derivative, AraCTP. This process is influenced by multiple

factors, composed of transporter, phosphorelation, deaminating and competing metabolites.

Mechanisms of AraC

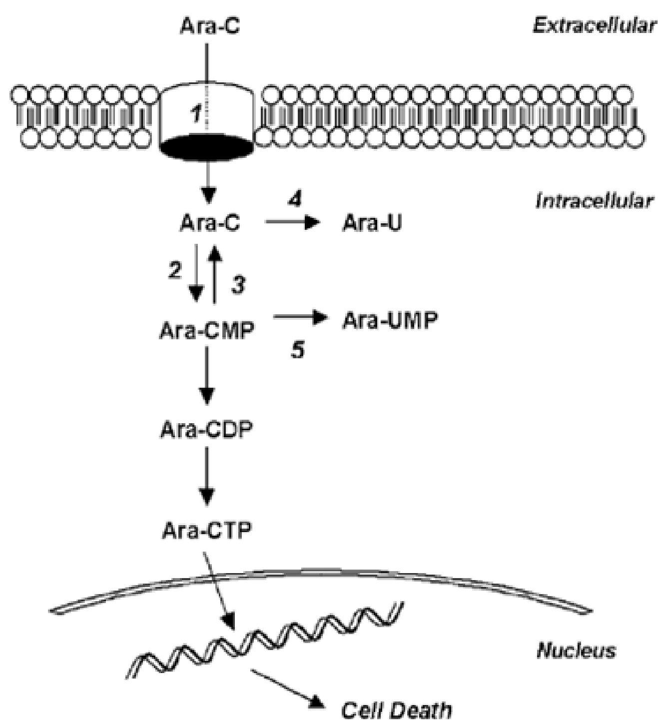


Figure 3.1: AraC metabolism within cells [2]

1. hENT1 (Human equilibrative nucleoside transporters 1)
2. dCK (Deoxy cytidine kinase)
3. PN I (Pyrimidine nucleotidase I)
4. CDA (Cytidine deaminase)
5. dCMPD (Deoxycytidylate deaminase)

AraC is mainly uptaked in cell by the human equilibrative nucleoside transporter 1 (hENT1)[49, 50]. Inside the cell, AraC is phosphorylates by deoxycytidine kinase (dCK) to form AraCMP. Deoxycytidine kinase is the rate limiting activation step of AraC. After that AraCMP is phosphorelates to AraCDP by deoxycytidylate kinase and finally it is phosphorelates into active metabolize form or

cytotoxic form, cytosine arabinoside triphosphate (AraCTP) by nucleotide diphosphate kinase. The AraCTP is compete with deoxycytidine triphosphate (dCTP) for incorporated into DNA. When incorporated, AraC blocks mechanism of DNA synthesis and after that the cell is subjected to program cell death. Pyrimidine nucleotidase I (PN-I) catalyzes the dephosphorylation of AraCMP, opposite the action of dCK. And cytidine deaminase (CDA) and Deoxycytidylate deaminase (dCMPD) is deaminate the cytosine base convert AraC to Ara-U and AraCMP to Ara-UMP respectively [2, 21, 27, 46, 51, 52]. Inactivation of AraC and AraCMP by these deaminating enzymes decrease the amount of active metabolized enzyme (AraCTP) that is cytotoxic effects of AraC [2, 53-55].

3.5.1 AraC mediated Cytotoxicity

3.5.1.1 Inhibition of DNA polymerase and DNA incorporation

The active metabolite of AraC, AraCTP, acts in two ways (a) inhibition of DNA polymerase (b) incorporated AraC into the DNA and causing block of DNA synthesis. Inhibition of DNA polymerase by AraC result as a weak competitive. The incorporation disrupts DNA elongation and induced to apoptosis [56].The accumulation and retention of AraCTP and incorporate into DNA have been shown to be the main factor of cytotoxic effect [57-60] .

3.5.1.2 AraC accumulation and retention

The accumulation of AraC, long retention and high corporation into DNA determine the sensitivity of cell in culture. The relationship between high accumulation of AraC and long retention in leukemic lymphoblast and the response rate and duration of reponse were shown by Rustum et al [61]. The retention of AraC was the critical factor in the response of patients to AraC treatment [62].

3.5.2 Pharmacokinetic of AraC

AraC is not administered orally, only by intravenous [63-65], because of the CDA level was high in liver and high in first-pass elimination. Male has faster clearanced than female and the clearance was correlated with the pretreatment white

blood cell blast count [66]. High dose of AraC was effective in remission induction in AML patients and lend to high drug level in cerebrospinal fluid of patients treated with regimen for central nervous system leukemia.

3.5.3 Side effect of AraC

Common side effect of AraC including thinned or brittle hair, headeach, weakness, loss of weight, and tingling in the hand or feet. The other sign is painful urination, black stools, diarrhea, pain in abdomen, bruising or bleeding, cough, fever, sore throat, nausea and vomiting, rash, yellowing of the skin or eye , pain in the joint, muscle weakness and blurred vision [67].

3.5.4 dCK gene

The dCK is located on chromosome 4q13.3, spanning over 34 kb and contain seven exons [7, 68]. dCK is the rate limiting enzymes that catalyzes the initial step of phosphorylation of deoxycytidine (dCyd) to deoxycytidine monophosphate (dCMP) and of many nucleoside analogue such as, AraC, gemcitabine, clofarabine, and other[68-71]. The studying about dCK and promoter in Chinese population, two promoter variants, -360 C>G and -201 C>T are associated with good clinical response but homozygous for wild type associated with poor drug response [7]. The reducing of dCK mRNA expression and deficiency of functional dCK has been associated with AraC resistance [72-75]. The relapsed ALL and AML patients showed decreasing of dCK mRNA expression [9]. In vitro expression of alternatively spliced form of dCK mRNA were detected only in leukemic blasts from resistant AML patients but not in leukemic blasts from sensitive AML patients [76]. Several cell lines, which lack of dCK activity were resisted to nucleoside analogue such as AraC or gemcitabine [75, 77-81].

3.5.5 CDA gene

The CDA gene is located on chromosome 1p36.2-p35 which encode enzymes cytidine deaminase, play an essential role in metabolism of antitumoural cytosine nucleoside analogues, such as AraC, aza-cytidine and 2’,2’-difluorodeoxycytidine. CDA is deaminase AraC to inactive form and the increasing level of CDA is correlated to the resistance to AraC both in vivo [82-84] and in vitro

study. The balance of level of CDA between activation and degrading enzymes is important in determine the AraC cytotoxicity. Two nonsynonymous single nucleotide polymorphisms are 79 A>C (Lys27Gln) ,and 208 G>A(Ala70Thr). The impact of these SNPs on AraC toxicity are difference in functional enzyme activity between wild type and variant CDA 208 G>A [19].

3.6 Genotyping Method

The majority of human variants are single nucleotide polymorphisms (SNPs), single base substitute of one nucleotide with another and the frequency is greater than 1% of general population, the SNPs is also a genetic marker for some diseases [85]. Single nucleotide polymorphism in drug metabolism can explain the differences of clinical outcome [20].Large scale of genotyping is crucial to identify the genetic marker and variation in drug response [86]. This knowledge, we are applied the study of genotyping for correlated with AraC toxicity.

3.6.1 Polymerase chain reaction -Restriction Fragment length polymorphism (PCR-RFLP) Analysis

The RFLP method has useful in screening for genetic mutation [87,88], this method involve restriction enzyme cut at restriction site of DNA sample divided into fragment of DNA. Follow by two steps were amplification of specific region of the interesting gene by PCR and digestion of the amplification of PCR by restriction enzyme. This method can divided genotype such as wild type and mutant sequence. The reaction for RFLP method was shown in figure 3.1

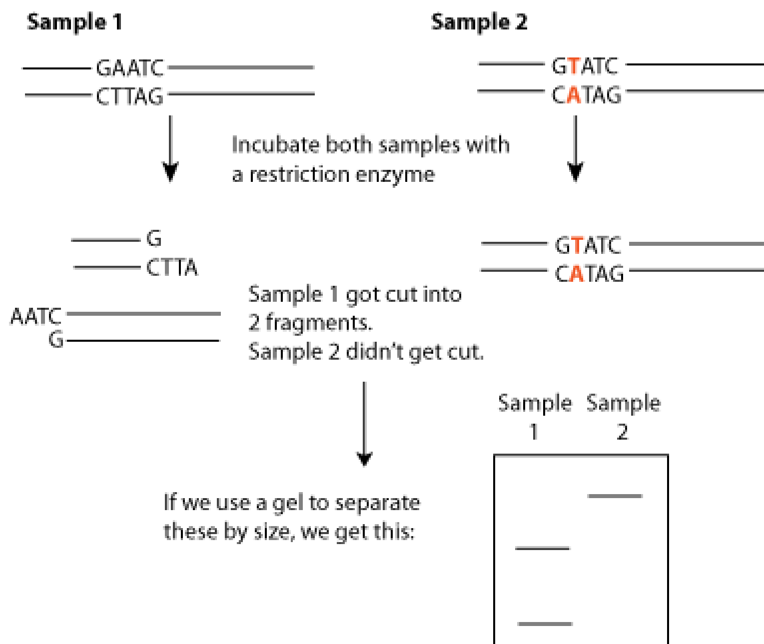


Figure 3.2 Reaction for RFLP technique

3.6.2 Amplification Refractory Mutation System (ARMS)

Amplification refractory mutation system (ARMS) was first described by Newton and colleages in 1989 as simple, reliable and non-isotopic technique [89] and has become a standard method to discriminated of alleles that differ as 1 bp. The basic of the system was that oligonucleotides with a mismatch 3' terminal residual primers in PCR condition. A standard ARMS PCR consists of two reactions (two tubes), one of primers was internal control for each reaction and the other primers for wide-type sequence or variant sequence. One of wild type and variant primers were differ at their 3'terminal residual and specific for each sequence of each genotype only. This method required four primers, two primer for internal control and each couple of primer for interesting site of DNA (ARMS primers), in one PCR reaction. Two of internal control primers that amplify a fragment of DNA some distance away from the site of the interesting site, so that they do not interfere with the amplification of DNA fragment produced by ARMS primers [90]. The control fragment acts as an indicator that the PCR condition was properly. The ARMS primer in the each reaction were specific to either the normal sequence or mutant sequence as require, these ARMS primers were

combine to produced the DNA fragment in the amplification reaction. The reaction for ARMS was shown in figure 3.2

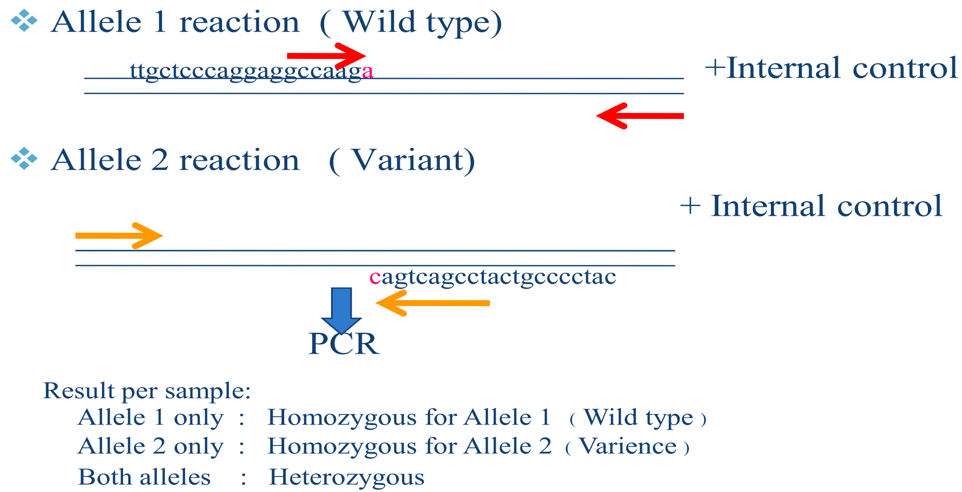


Figure 3.3 Principle for SNPs genotyping by ARMS

3.6.3 Direct DNA Sequencing

DNA sequencing is the method for determine sequence of DNA such as adenine, guanine, cytosine and thymine. The advantage of this method is used for biological research. Sanger devised this method base on dideoxynucleotide sequencing, utilizes 2’3’-dideoxynucleotide triphosphate (ddNTPs) molecular that differ from dideoxynucleotides by having a hydrogen atom attached to the 3’carbon rather than an OH group. These molecules terminate elongated DNA because they can not form phosphodiester bond with the deoxynucleotide (Figure 3.3).

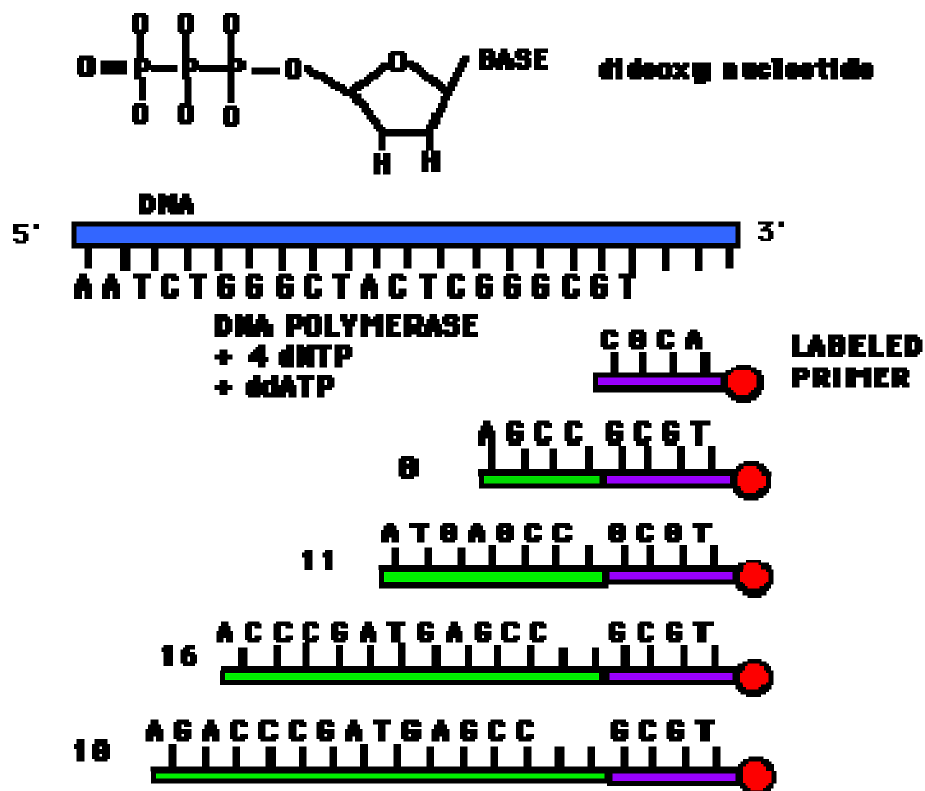
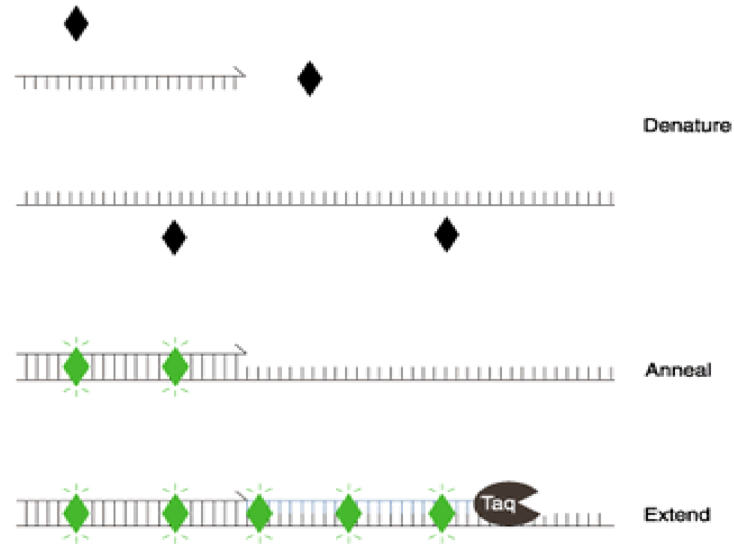


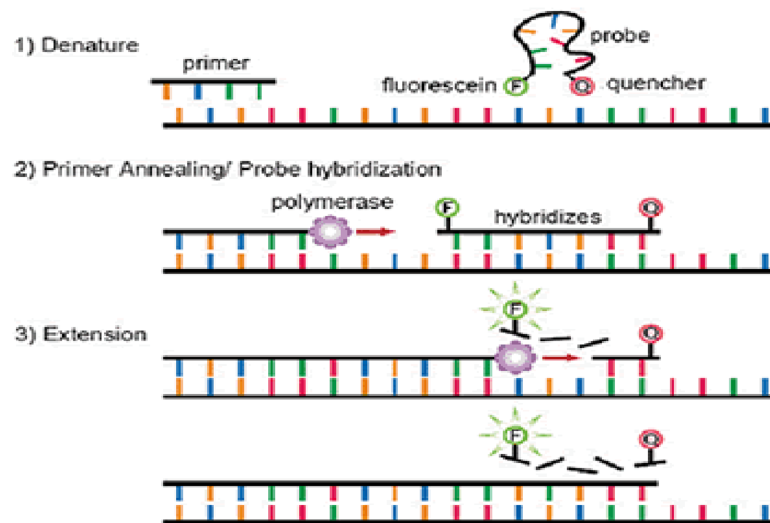
Figure 3.4 Reaction of DNA sequencing

3.7 Quantitative real-time PCR (RQ-PCR)

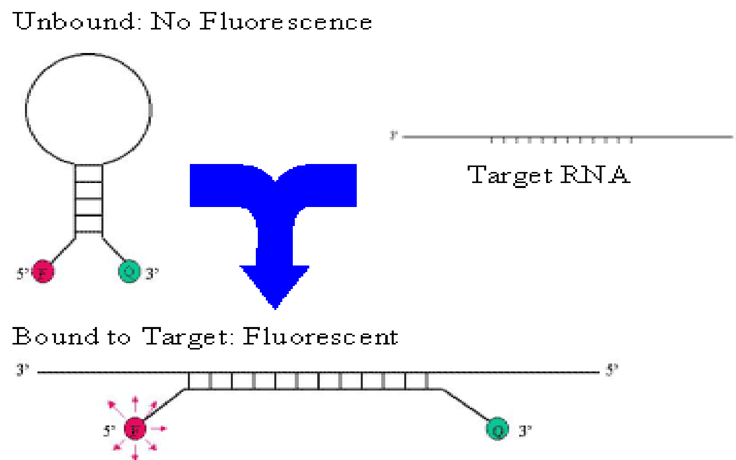
Real time PCR is a real time and on line analysis of PCR products during amplification in a closed system without post PCR processing. Real time PCR is based on detection and quantitation of fluorescence reporter. Many fluorescence using for real time PCR such as SYBR Green which base on double stand DNA (dsDNA) binding dye and other fluorescence base on Fluorescence Resonance Energy Transfer (FRET) compose of three probes such as Molecular beacon probe, TaqMan Probe, and Hybridization probe.(Figure 3.4)



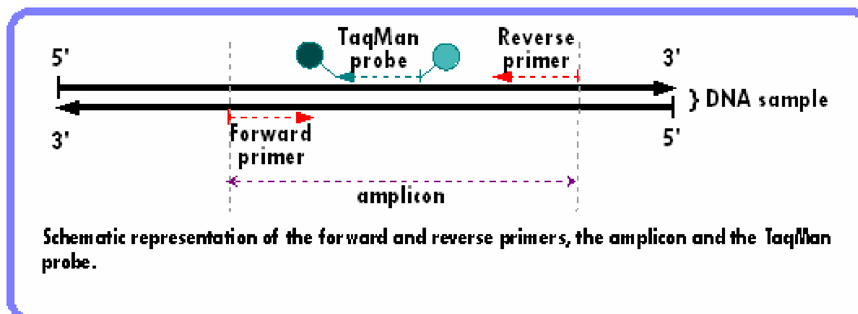
SYBR Green base on double stand DNA binding dye



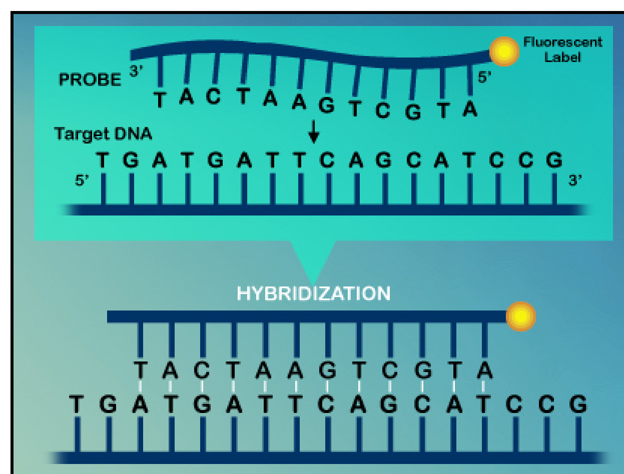
Probe base on FRET



Beacon probe



Taqman probe



Hybridization probe

Figure 3.5 Fluorescence for Real time PCR

The FRET technique for detecting in real time PCR is higher specific than dsDNA binding dye (SYBR Green) but the cost of SYBR Green is lower than several probe for using FRET technique. The reaction of SYBR Green was acted in PCR amplification, SYBR Green was binding with minor groove of DNA and then fluorescence can detected.

Threshold cycle (C_T) is the important parameter for quantitative cycle number which the fluorescence generated within a reaction crosses the threshold. The C_T value assigned to a particular well thus reflect the reaction of number of amplicon accumulated. The C_T value use to calculate relative gene expression which chosen internal control as more abundant and remain constant among the samples such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 18S RNA and β-actin, for normalized quantitative of mRNA expression.

CHAPTER IV
MATERIALS AND METHODS

4.1 Biological samples

Patient Samples

Peripheral blood and bone marrow samples were collected from Thai childhood acute lymphoblastic patients at Division of pediatric Hematology-Oncology unit, Department of Pedriatic, Ramathibodi Hospital. All have been previously diagnosed as acute leukemia by morphological, cytochemical, and immunophenotyping methods. These included of 94 peripheral blood samples from Thai childhood acute lymphoblastic patients who was treated by Rama ALL protocol and had complete remission induction phase where as the 44 bone marrow samples were collected from untreated ALL patients for RNA isolation for mRNA expression study.

Control Samples

The 100 peripheral blood samples from donors of Blood Bank Division, Department of Pathology, Ramathibodi Hospital were included in the study as a control group.

The present study was approved by the ethical committee of Faculty of Medicine, Ramathibodi Hospital.

4.2 Materials

4.2.1 Instruments and laboratory supplies

- Bench-top microcentrifuge (Sprout Company, China)
- Autopipett 0.5-2 µl, 10 µl, 20 µl, 100 µl, 200 µl and 1000 µl (Gilson Company, France)
- Horizontal gel electrophoresis (Bio-Rad Company, USA)
- UV- transluminator with camera computer processing (Dolphin, SciTech Pty LTD., Australia)

- Power supply (Bio-Rad Company, USA)
- Thermocycler (Eppendorf Mastercycler gradient, Eppendorf Company, USA)
- Light cycle instrument (Bio-Rad Company, USA)
- Refrigerater -4°C (Sanyo Company, Japan)
- Freezer -20°C (Sharp Company, Japan)
- Freezer -80°C (Jouan Company, France)
- Microcentrifuge tube 1.5ml and 0.5 ml in sizes (Axygen Company, USA)
- Pipette tips 2-10 µl (Axygen :white)
  20-200 µl (Axygen :yellow)
  1000 µl (Axygen :blue)
- Light cycle capillaries (Bio-Rad Company, USA)
- Microwave (Hitachi Company, Japan)
- Glassware (Duran Company, Germany)
- Vortex mixer

4.2.2 PCR Reagents KIT

- Taq DNA Polymerase Enzyme (Invitrogen Company, Brazil)
- 10X PCR buffer composed of 200mM Tris-HCl (pH 8.4) and 500 mM KCl
- 50 mM $MgCl_2$
- Deoxynucleotide triphosphate (dNTP) 10µM each of 2’deoxyadenoside 5’triphosphate (dATP), 2’deoxytymidine 5’triphosphate (dTTP) , 2’deoxycytidine 5’ triphosphate (dCTP) and 2’deoxyguanosine 5’triphosphate (dGTP)
- Oligonucleotide primers (Fermentus Company, USA)(Table 4.1)

4.2.2.1 Reagent Preparation for dCK gene PCR position -360C>G, -201C>T

From PCR reagent kit,2.5µl 10 X PCR buffer, 1.5µl 50 mM $MgCl_2$, 1.2 µl 10mM dNTPs, 0.5 µl 10µM of each of the forward and reverse primers for DCK gene -360C/G , -201C/T (Table 4.1), 1 µl DMSO ,0.3 µl 5U/µl of Taq DNA Polymerase Enzyme and 15.5 µl sterile dH_2O was added to a final volume of 23 µL of reaction mixture.

4.2.2.2 Reagent Preparation for CDA gene PCR position CDA208G>A

From PCR reagent kit, 2.5 µl 10 X PCR buffer, 1.5µl 50mM $MgCl_2$, 1.2µl 10 mM dNTPs, 0.5µl 10 µM of each of the forward and reverse primers for CDA gene position 208G/A (Table 4.1), 0.1 µl 5U/µl of Taq DNA Polymerase Enzyme, and 17.7 µl sterile dH_2O was added to a final volume of 24 µl of reaction mixture.

Table 4.1 : Primers for PCR

Gene	**Primer**	**5'→3'Sequence**	**Product Size**(bp)
dCK	Forward Reverse	CTG CAG GTG ACG CCC TCT GGG TGG CCA TTC CTT AGT CT	469
CDA : 208 G>A	Forward Reverse	AAC ACA CGC AAC AGG AAG TG ATT GTT GCA ACC TGG CTT TC	200

4.2.2.3 Reagent Preparation for Wild Type CDA 79A>C

From PCR reagent kit, 2.5 µl 10X PCR buffer, 1.5µl 50mM $MgCl_2$, 1.2 µl 10mM dNTPs, 1.2 µl 10 µM of each of the Internal control (ACTB) forward and reverse primers, 0.3 µl 10 µM of each of the forward Primer (wild type) and reverse Primer (wild type) (Table 4.2) and 0.1 µl 5 U/µl of Taq DNA Polymerase Enzyme and 15.7 µl sterile dH_2O to a final volume of 24 µl of reaction mixture.

4.2.2.4 Reagent Preparation for Variant Type CDA 79A>C

From PCR reagent kit , 2.5µl 10 X PCR buffer, 0.75 µl 50mM $MgCl_2$, 1.2 µl 10mM dNTPs, 1.2 µl 10 µM of each of the Forward Primer (internal control, ACTB) and reverse Primer (internal control, ACTB), 0. 5 µl 10 µM of each of the forward Primer (variant type)and reverse primer (variant type) (Table 4.2) and 0.1 µl 5 U/µl Taq DNA Polymerase Enzyme and 16.05 µl sterile dH_2O to a final volume of 24µl of reaction mixture.

Table 4.2 Primers for ARMS

Gene	Primer	5'→3'Sequence	Product Size(bp)
CDA 79 A>C	WT-F	TTG CTC CCA GGA GGC CAA GA	194
	WT -R	CTT GCC ACT GCC TGT GCC T	
	Va-F	ACC AAC ATG GCC CAG AAG CG	105
	Va -R	GTA GGG GCA GTA GGC TGA CTG	
Beta-actin (ACTB)	Forward	CTAACACTGGCTCGTGTGACAAG	751
	Reverse	CATGCCTGAGAGGGAAATGAG	

4.2.3 Reagents for agarose gel electrophoresis

- 10X TBE buffer composed of 0.89 M Trizma base, 0.89 M Boric acid and 0.02 M EDTA
- Orange DNA loading dye composed of 10 mM Tris-HCl (pH7.6) 0.15% orange G, 0.03% xylene cyanol, 60% glycerol and 60 mM EDTA (Fermentus company, USA)
- Molecular weight marker (New England Bio Labs Inc company, USA)

4.2.3.1 Agarose gel preparation

A. 2% agarose gel for PCR product

Agarose gel was weigh for 0.4 gm in dH_2O 20 ml, then melted on microwave oven. Melting agarose gel was poured in tray and insert comb too and make sure no bubble in gel, to leaved at room temperature about 40 minutes until agarose gel was cool ready for using.

After gel ready for used, withdraw the gel comb and then placed agarose gel to tank of electrophoresis. Fill the 1X buffer in tank of electrophoresis for cover it about 1 mm.

B. 3% agarose gel for fragment of digestion.

Method for prepared agarose gel was previous describes on above but agarose weigh for 0.6 gm in dH_2O 20 ml.

4.2.4 Reagents for enzymatic restriction fragment length polymorphism (RFLP) technique

4.2.4.1 1X Buffer Tango^{TM} for ***BsrB I, Ehe I*** composed of 33 mM Tris-acetate (pH 7.9) 10 mM magnesium acetate, 66 mM potassium acetated, 0.1 mg / ml BSA (Fermentus Company, USA)

4.2.4.2 1X NEB buffer for *Rsr II* composed of 50 mM potassium acetated, 20 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate and 1mM DTT (New England BioLabs Inc company, USA)

4.2.4.3 Restriction Enzyme (Table 4.3)

Table 4.3 Restriction enzymes for PCR-RFLP

Gene	Polymorphism position	Restriction enzyme
dCK	-201 C>T	*BsrBI* CCG↓CTC
	-360 C>G	*Ehe I* GGC↓GCC
CDA	208 G>A	*Rsr II* CG↓GWCCG

4.2.5 ImProm-II™ Reverse Transcription System Reaction Kits (Promega Company, USA)

- 5 X Buffer Reverse Transcription composed of 10mM Tris-HCl (pH 9.0), 50mM KCl and 0.1% Triton® X-100
- 25 mM $MgCl_2$
- dNTP : 10 μM each of dATP, dTTP, dCTP, dGTP
- Random Primer
- Recombinant RNasin Ribonuclease Inhibitor
- AMV Reverse transcriptase
- Nuclease - free water

4.2.5.1 Reagent Preparation from ImProm-II^{TM} Reverse Transcription System Reaction Kits for reverse transcription

From ImProm-II^{TM} Reverse Transcription System Reaction Kits, 4 µl of 5X Buffer Reverse transcription buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 10 µM dNTPs Mixture, 0.5 µl of Recombinant RNasin $^{®}$ ribonuclease inhibitor, 0.6 µl of AMV Reverse transcriptase, 1 µl of Random primer and Nuclease free water dH_2O was added into a final volume of 20µl of reaction mixture.

4.2.6 iQ^{TM} Supermix kit (Bio-Rad Company, USA)

- 1X Supermix composed of 50 mM KCl, 20 mM Tris-HCl 0.8mM dNTPs, iTaq DNA polymerase, 3 mM $MgCl_2$ and stabilizers
- DNA template
- dH_2O, PCR grade (to adjust the final reaction volume)
- 10 µM Forward and reverse primers for each dCK gene, CDA gene , ACTB gene (Table 4.4)

4.2.6.1 Reagent Preparation from iQ^{TM} Supermix kit for Real time PCR

From iQ^{TM} Supermix kit**,** 1 µl (50 ng) of cDNA template was Master mix with 0.5 µl 10µM each of Forward and Reverse primers(Table 4.4), 12.5 µl 0.5 X iQ^{TM} Supermix kit (Bio-Rad Company, USA)) and 10.5 µl sterile dH_2O in total volume 25µl.

Table 4.4 : Primers for Real-time PCR

Gene	Primer	5'→3' Sequence	Product Size(bp)
dCK	Forward Reverse	CTCTGGGCCGCCACAA CCCTTCGATGGAGATTTTCTTG	109
CDA	Forward Reverse	CTGCCTTGGGACTTAGAACAC TGCTCGGAACAGGATAGAACC	134
Beta-actin (ACTB)	Forward Reverse	GATCGGCGGCTCCATCCTG GACTCGTCATACTCCTGCTTGC	75

4.3 Methods

4.3.1 DNA Genotyping

4.3.1.1 Primer design

Four single nucleotide polymorphisms (SNPs) of two genes were studied (i) CDA 79 A>C and 208 G>A (ii) dCK -360 C>G and -201 C>T. The primers were designed as follows: SNPs positions 79 A>C and 208 G>A using Primer 3 software, -360 C>G and -201 C>T follow M. Joerger et al technique [8], ACTB primer by Primer express software (design by Applied Biosystems)and real time PCR primers by Beacon software (design by Bio-Rad, USA). All primers were designed in balance between specificity and efficiency of amplification. Specificity was control by length of primers and anealling temperature of PCR reaction, length of primer have oligonucleotide between 18-24 bases tend to very specific. The 3' terminal position of primer would were G or C alleles, G+C base composition should be 50-60 % and calculated melting temperature between 50-80 ºC.

Step for design primers : entry to primer 3 website and past the sequence nucleotide of gene (fasta format) on the fix box. Click for left and right primer, set parameter such as primer size, number to return or used as default of program and then pick primer for get the primers.

The primers were tested for possible repetitive sequence, and aligned with the sequence databases at the National center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) family of

program, to check for primer specific. To check for self-dimer hetero-dimer and hair pin loop OligoAnalyzer program was used.

4.3.1.2 Molecular genetic study

94 peripheral blood samples from treated Thai childhood acute lymphoblastic patients and control group were performed for DNA isolation by phenol chloroform standard protocol (19) and all samples were DNA already. Then genomic DNA was amplified by polymerase chain reaction, the primers used are shown in Table 4.1, followed by enzymatic restriction fragment length polymorphism (RFLP) technique for CDA 208 G>A and dCK -360 C>G, -201 C>T where as the ARMS PCR base technique for CDA 79 A>C by using two primer :primer for internal control and primer for detected of wild and variant type (Table 4.2) before electrophoresis on 3% (w/v) agarose gel and the genotypes separated. Sequencing was used to confirm some case of samples.

Method for polymerase chain reaction

A. Method for PCR amplification of dCK -360 C>G, -201 C>T

2 µl (100 ng) of genomic DNA was put into master mixed with reagent preparation for dCK-360C>G, -201C>T that composed of 2.5µl 10 X PCR buffer, 1.5µl 50 mM $MgCl_2$, 1.2 µl 10mM dNTPs, 0.5 µl 10µM of each of the forward and reverse primers for dCK-360 C>G, -201 C>T (Table 4.1), 1 µl DMSO,0.3 µl 5U/µl of Taq DNA Polymerase Enzyme and 15.5 µl sterile dH_2O was added to a final volume of 25 µL of reaction mixture.

PCR amplification was performed with the Thermocycler (Eppendorf Mastercycler gradient, Eppendrof company USA).The first round PCR reaction was performed using pre-denaturation step of 5 min at 95 °C followed by 20 denaturation cycles of 1 min at 95 °C, annealing step of 1 min at 61 °C and an extension at 72 ºC for 1 min. This was followed by 20 cycles of denaturation of 1 min at 95 °C, annealing 0f 1 min at 55 ºC and extension at 72 ºC for 1 min and post extension at 72 ºC for 10 min.

The enzymatic restriction fragment length polymorphism (RFLP) technique for dCK -360 C>G, -201 C>T

8.5µl of the PCR products was digested by 0.5 µl (5 unit) *EheI* restriction enzyme for dCK-360 C>G, then added 1 µl 1X Buffer Tango^{TM} to a final volume of 10 µL of reaction mixture whereas the other 8.5µl of the PCR products was digested by 0.5 µl (5 unit) *BsrBI* restriction enzyme for dCK gene position -201 C>T, followed by 1 µl Buffer Tango^{TM} to a final volume of 10 µL of reaction mixture. Each of the reaction mixture was incubated at 37 ºC overnight.

Electrophoresis on 3% agarose gel

10 µl of digested PCR products and 2 µl of gel loading dye solution was added, mixed well. Then 4 µl of molecular weight marker was added to separate difference size of fragment DNA.

This mixture were electrophoresed on 3 % agarose gel for 60 minutes 100 V (5 V/cm) and then stained with ethidium bromide[19].Bands of PCR product was directly to visualized by UV trans-illumination (Dolphin,SciTech Pty LTD,Australia) and confirmed some case by sequencing method ABI DNA Sequencer 3730 XL genetic analyzer (Perkin, Elmer Applied Biosystems, USA).

B. Method for PCR amplification of CDA 208 G>A

1 µl (100 ng) genomic DNA was put into master mix with 2.5 µl 10 X PCR buffer, 1.5µl 50mM $MgCl_2$, 1.2µl 10 mM dNTPs, 0.5µl 10 µM of each of the forward and reverse primers for *CDA*208G>A (Table 4.1), 0.1 µl5U/µl of Taq DNA Polymerase Enzyme, and 17.7 µl sterile dH_2O was added to a final volume of 25 µl of reaction mixture.

PCR amplification was performed with the Thermocycler (Eppendorf Mastercycler gradient, Eppendrop company, USA). The PCR reaction was performed using PCR reaction was performed using pre denaturation step of 5 min at 95 °C followed by 35 denaturation cycles of 1 min at 95 °C , annealing of 1 min at 60 ºC and extension at 72 ºC for 1 min and post extension at 72 ºC for 10 min.

Enzymatic Restriction Fragment Length Polymorphism (RFLP) technique for CDA 208 G>A polymorphism

5 µl of the PCR products was digested by 0.5 µl (5 unit) *RsrII* restriction enzyme, 1 µl of 1X NEB buffer 4, and 3.5 µl dH_2O to a final volume of 10 µL of reaction mixture. Then the reaction mixture was incubated at 37 ºC overnight.

Electrophoresis (3 % Agarose Gel)

10 µl of Digested PCR products and 2 µl of loading dye solution was added and mixed well. Then 4 µl of molecular weight marker was added to separate difference size of fragment DNA. This mixture were electrophoresed on 3 % agarose gel for 60 minutes,100 V (5 V/cm) and then stained with ethidium bromide[19].Bands of PCR product was directly to visualized by UV trans-illumination (Dolphin, SciTech Pty LTD, Australia). and confirmed some case by Sequencing method ABI DNA Sequencer 3730 XL genetic analyzer (Perkin, Elmer Applied Biosystems, USA).

C.ARMS-PCR technique for Genotyping 0f CDA79 A>C polymorphism

Each sample was tested in two conditions,(i) condition for wild type (WT, normal homozygous) was carry out with internal control primers (fragment of PCR product was 751 bp) and CDA 79 WT forward primers (194 bp for wild type). (ii) condition for variant (mutant homozygous) internal control primer (fragment of PCR product was 751 bp) and CDA 79 variant primers (105 bp for variant type). Internal control primer and CDA 79 wild type and variant primers as show at table 4.2 and fragment of them were shown respectively.

wild type genotyping

1 µl (100 ng) genomic DNA was put into master mix with 2.5µl 10 X PCR buffer, 1.5 µl 50mM $MgCl_2$, 1.2 µl 10mM dNTPs, 1.2 µl 10 µM of each of the Forward Primer (internal control, ACTB) and reverse Primer (internal control, ACTB), 0. 3 µl 10 µM of each of the forward primer (variant type)and reverse primer (variant type) (Table 4.2) and 0.1 µl 5 U/µl Taq DNA Polymerase Enzyme and 15.7 µl distilled water (DW) to a final volume of 25µl of reaction mixture.

Variant type genotying

1 µl (100 ng) genomic DNA was put into master mix with 2.5µl 10 X PCR buffer, 0.75 µl 50mM $MgCl_2$, 1.2 µl 10mM dNTPs, 1.2 µl 10 µM of each of the Forward Primer (internal control, ACTB) and reverse Primer (internal control, ACTB), 0. 5 µl 10 µM of each of the forward Primer (variant type) and reverse primer (variant type) (Table 4.2) and 0.1 µl 5 U/µl Taq DNA Polymerase Enzyme and 16.05 µl DW to a final volume of 25µl of reaction mixture.

PCR for wild and variant genotyping was performed with the Thermocycler (Eppendorf Mastercycler gradient, Eppendrof company, USA). The PCR reaction was performed using pre-denaturation step of 5 min at 94 °C followed by 35 denaturation cycles of 30s at 94 °C, annealing of 30s min at 68ºC and extension at 72 ºC for 1 min and post extension at 72 ºC for 10 min.

Electrophoresis (2% Agarose gel)

10 µl of digest PCR products and 2 µl of orange gel loading dye was added and mixed well. Then 4 µl of molecular weight marker was added to separate difference size of fragment DNA.

This mixture were electrophoresed on 2 % agarose gel for 45 minutes at 100 V (5 V/cm) for PCR product and then stained with ethidium bromide [19]. Bands of PCR product were directly visualized by UV trans-illumination (Dolphin, SciTech Pty LTD, Australia) and confirmed some case by Sequencing method ABI DNA Sequencer 3730 XL genetic analyzer (Perkin, Elmer Applied Biosystems, USA).

4.3.2 mRNA Expression study

All samples were transformed into RNA by extracted from bone marrow by using Trizol Reagent [52]. The RNA was changed to complementary DNA (cDNA) by Improm II™ reverse transcription kit. Quantitative PCR was conducted using cDNA as template and primers designed from CDA, dCK gene according to manufacture's instruction (Beacon software).Endogenous housekeeping gene encoding for beta actin (ACTB) was used as an internal standard (reference) for mRNA expression study by quantitative PCR (RQ-PCR) technique.

4.3.2.1 Reverse transcriptase PCR (RT-PCR) technique

3 µl of isolated RNA was incubated at 70 ºC for 10 minutes and then RNA was reverse transcriped using reagent preparation from Improm II^{TM} reverse transcription kit condition, that composed of 4 µl of 5X Buffer Reverse transcription buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 10 µM dNTPs Mixture, 0.5 µl of Recombinant RNasin ® ribonuclease inhibitor, 0.6 µl of AMV Reverse transcriptase, 1 µl of Random primer and Nuclease free water was added into a final volume of 20 µl of reaction mixture.

The reaction mixture was incubated at 25ºC for 10 minutes, 42ºC for 1 hour and 70ºC for 15 minutes.

4.3.2.2 Quantitative (Real time) PCR (RQ-PCR) and melting curve analysis

1 µl (50 ng) of cDNA template was put into Master mix with 0.5 µl 10µM each of forward and reverse primers (Table 4.4), 12.5 µl 0.5 X iQ^{TM} Supermix kit (Bio-Rad Company, USA)) and 10.5 µl sterile dH_2O in total volume 25µl

This reaction mixture was load into 96 well plates covered with plastic plate and placed in the light cycle instrument with initial denaturation step at 95°C for 3 min for 1 cycle; followed by 35 amplification cycles, each comprising denaturation 95 ºC for 10s, annealing and extension at 65 ºC for 30s. After amplification, melting curve analysis was performed by cooling from 60-95 ºC, holding at 95 ºC for 1 min, 60 ºC for 1 min and then heating slowly at rate of 0.5 ºC/10s.

The RQ-PCR was performed in duplicate for each sample and Non template control (NTC) were run as negative control in pararell to determine amplification efficiency within each experiment. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimmers [52, 91].

All PCR performed with equal efficiency, the relative mRNA expression levels of CDA and dCK for each patient can directly be normalized with their own ACTB expression. The relative mRNA expressions of each gene were calculated by iQ5 software as state below.

$$\text{Normalized Expression}_{\text{sample(gene x)}} = \frac{\text{Relative Quantity}_{\text{sample(gene x)}}}{(\text{Rel Quant}_{\text{sample (Ref 1)}} * \ldots \text{Rel Quant}_{\text{sample (Ref n)}})}$$

4.3.3 Statistic analysis

Statistical analysis were used to evaluated the associated between genotypes and toxicity of AraC, evaluated the allele of each gene associated with toxicity and compared between the average of mRNA expression.

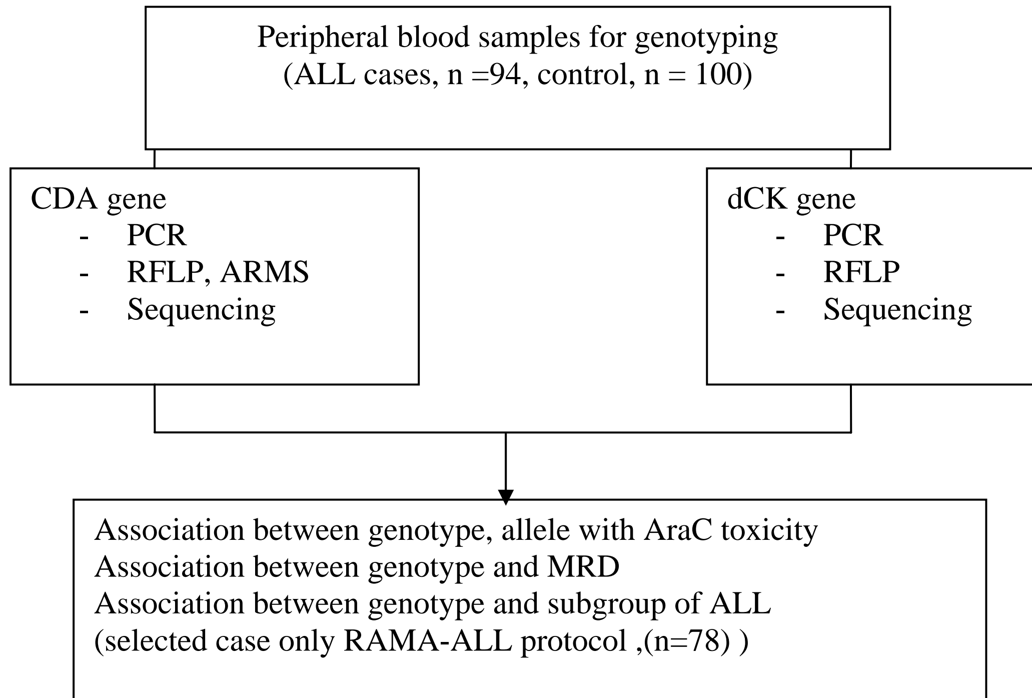
Correlation between genotype and toxicity, allele of gene and toxicity were tested in association with some variable such as, absolute neutrophil count (ANC), hemoglobin (Hb), platelet count (Plt), fever, mucositis, diarrhea, delay of treatment (delay) and febrile neutropenia (FN). Statistic were performed using Chi-square test, Fishers exact test, Odd ratio (OR), 95% confidence intervals (95% CI) were calculated.

Correlation between minimal residual disease (MRD) and genotype was tested in association by Fishers exact test, Chi-square test, 95% confidence intervals (95% CI) were calculated.

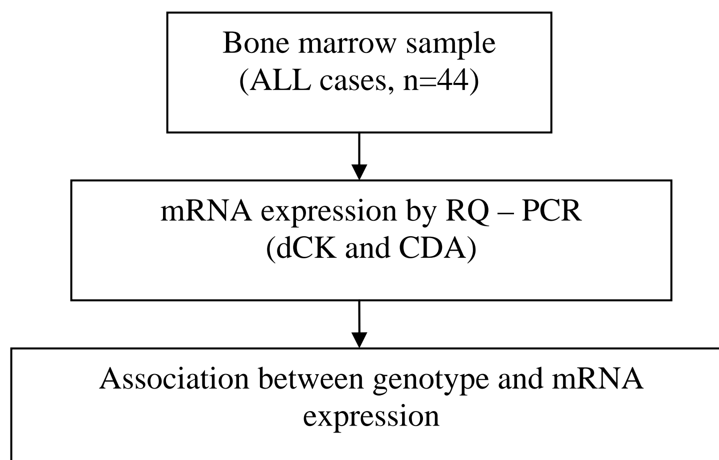
The mRNA expression of each gene were compared by Mann-Whitney U test, using 95% confidence intervals (95%CI) were calculated.

Flowchart

1. Genotypic analysis



2. mRNA Expression (CDA, dCK)



CHAPTER V
RESULT

5.1 The Clinical data of ALL patients and controls

The ALL patients were diagnosed at Division of Hematology–oncology, Department of Pedriatrics, Ramathibodi Hospital, Faculty of Medicine Mahidol University. Total of 94 patients composed of 54 males and 40 females (median age 5.8 years). The ALL peripheral blood samples were subtypes by immunophenotypic technique as 56 cases of early pre B ALL, 16 cases of pre B ALL, 1 case of B cell ALL, 10 cases of T-ALL, 2 cases of mixed lineage leukemia, 2 cases of relapse ALL, 4 cases of lymphoma and 3 cases of unidentified subtype

5.2 Genotyping

5.2.1 The establishment of amplification

A: dCK gene (-360 C>G, -201 C>T)

The lane 1-lane 6 (at 469 bp) were each sample amplification of *DCK* by PCR method as shown in figure 5.1

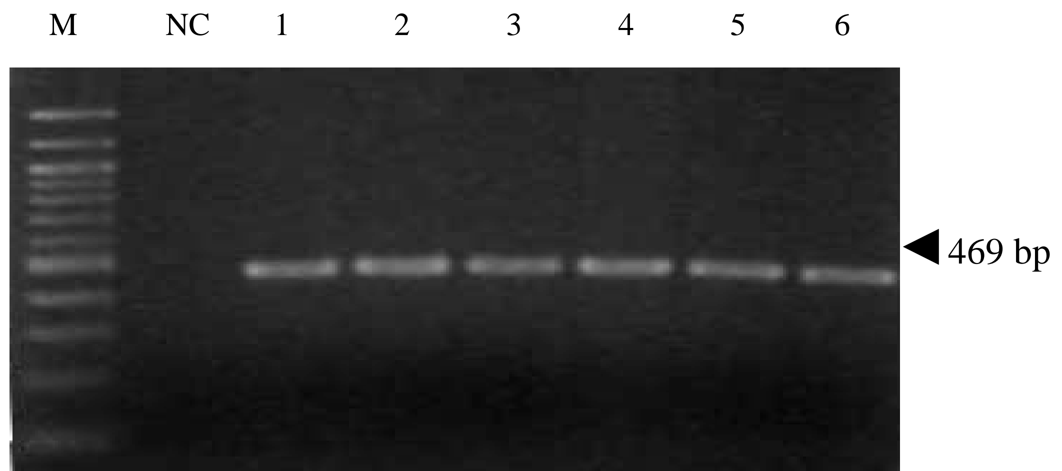


Figure 5.1 The amplification of dCK gene showed DNA ladder (M) was used as molecular marker. NC for negative control and lane 1-lane 6 were each sample amplification of dCK.

B. CDA 208 G>A

The lane 1-lane 13 (at 200 bp) were each sample amplification of *CDA* 208 G>A by PCR method as showed in figure 5.2

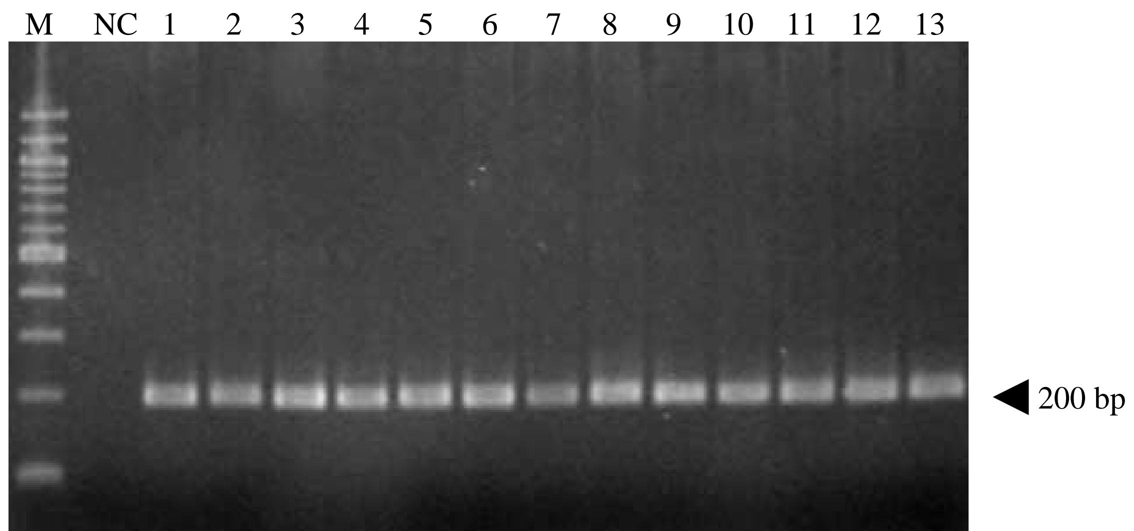


Figure 5.2 The amplification of CDA 208 G>A showed DNA ladder (M) was used as molecular marker. NC for negative control and lane1-lane 13 were each sample amplification of CDA 208 G>A .

5.2.2 Digestion with restriction enzyme

5.2.2.1 PCR-RFLP detecting for dCK (-360 C>G,-201 C>T)

For dCK position -360 C>G ,wild-type allele was cleavaged oligonucleotides by *EheI* at site 5'GGC↓GCC 3' as three fragments 93 bp, 100 bp and 276 bp. Variant allele was cleavaged as two fragments size as 93 bp and 376 bp and heterozygous was cleavaged as four fragments size as 93 bp, 100 bp, 276 bp and 376 bp. The fragments size 93 bp and 100 bp were very near and showed as one fragment by electrophoresis (figure 5.3). Lane 1 was the amplification of dCK gene. Lane 2, 3 and 4 were positive control for digestion of *EheI* of each genotype, wild type, heterozygous and variant type respectively. Another lane ,lane 5-lane 8 were the sample digestion of dCK -360 C>G lane 5 and lane 6 were wild type (CC genotype), showed three fragments size as 93 bp, 100 bp and 276 bp, lane 7 was heterozygous

(CG genotype) showed four fragments size as 93 bp, 100 bp, 276 bp and 376 bp and lane 8 was variant (GG genotype) showed two fragments size as 93 bp and 376 bp. (figure 5.3)

For dCK position -201 C>T, the wild type allele could not cleavaged by *BsrBI* whereas variant allele was cleavaged by *BsrBI* at site 5' CCG↓CTC 3' as two fragments size as 210 bp and 259 bp. So heterozygous of this SNP was created as three fragments size as 210 bp, 259 bp and 469 bp. The digestion of restriction enzyme of dCK -201 C>T were shown in figure 5.4. Lane 1 was the amplification of dCK. Lane 2, 3 and 4 were positive control for digestion of *BsrBI* of each genotype, wild type, heterozygous and variant type respectively. Another lane, lane 5-lane 8 were the sample digestion of dCK -201 C>T, lane 5 and lane 6 were wild type (CC genotype) showed one fragment size as 469 bp, lane 7 was heterozygous (CT genotype) show three fragments size as 210 bp, 259 bp and 469 bp and lane 8 was variant (TT genotype) showed two fragments size as 210 bp and 259 bp. (figure 5.4)

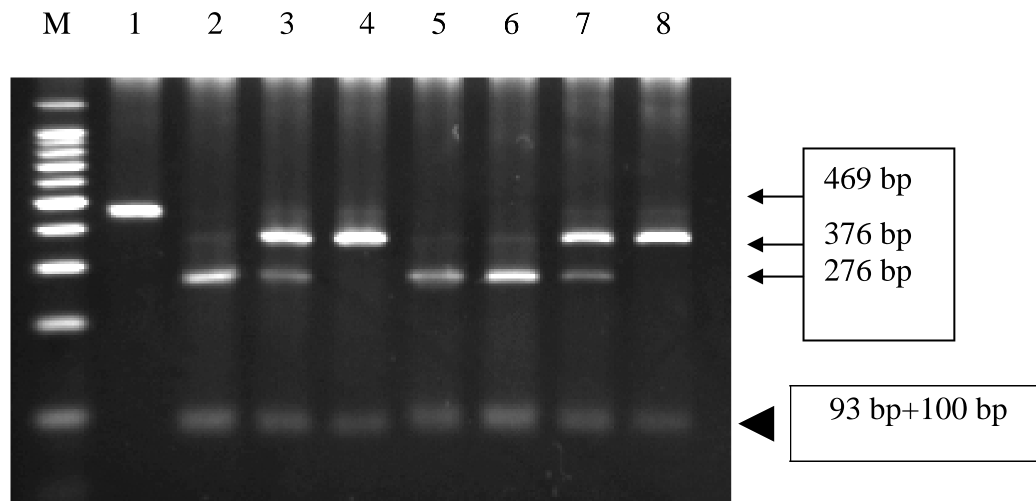


Figure 5.3 The restriction fragment of dCK -360 C>G. DNA ladder (M) was used as molecular marker. Lane 1 was the amplification of dCK gene. Lane 2, 3 and 4 were positive control for digestion of *EheI* of each genotype, wild type, heterozygous and variant type respectively. Another lane, lane 5-lane 8 were the samples digestion of dCK -360 C>G, lane 5 and lane 6 were wild type (CC), lane 7 was heterozygous (CG) and lane 8 was variant (GG).

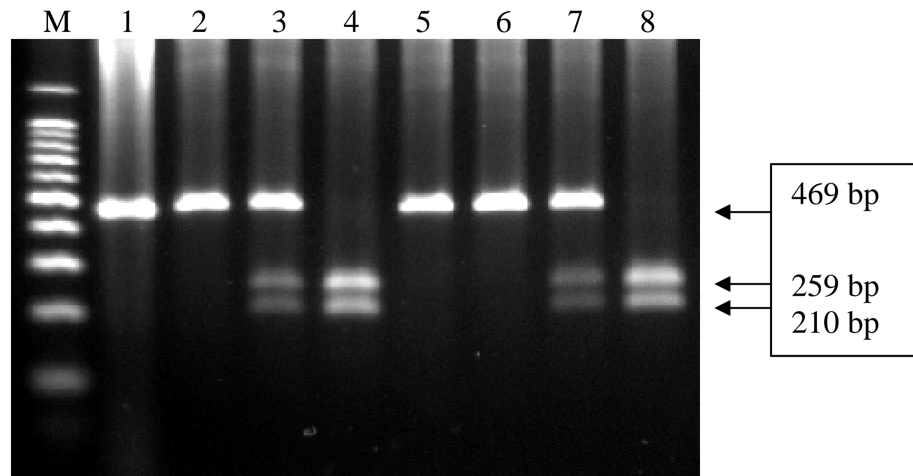


Figure 5.4 The restriction fragment of dCK -201 C>T showed DNA ladder (M) was used as molecular marker. Lane 1 was the amplification of dCK, Lane 2, 3 and 4 were positive control for digestion of *BsrBI* of each genotype, wild type, heterozygous and variant type, respectively. Another lane, lane 5-lane 8 were the samples digestion of dCK -201 C>T, lane 5 and lane 6 were wild type (CC), lane 7 was heterozygous (CT) and lane 8 was variant (TT)

5.2.2.2 PCR-RFLP detecting for CDA 208 G>A

The PCR amplification of wild type allele was cleavaged *by RsrII* at site 5'CG↓GWCCG 3' that does not present in variant allele. The digestion of restriction enzyme of CDA 208 G>A were shown in figure 5.5. Lane 1 was the amplification of CDA 208 G>A, lane 2 was positive control for digestion of *RsrII*. Lane 3 and lane 4 were samples digestion of CDA 208 G>A, they were wild type (GG genotype) showed fragments size as 86 bp and 114 bp.

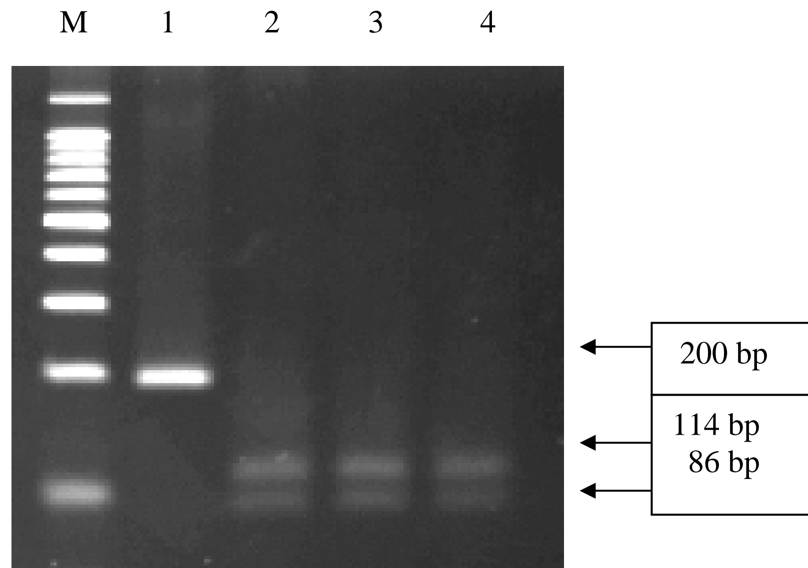


Figure 5.5 The restriction fragments of CDA 208 G>A showed DNA ladder (M) was used as molecular marker. Lane 1 was the amplification of CDA 208 G>A, lane 2 was positive control for digestion of *RsrII*. Lane 3 and lane 4 were as wild-type (GG genotype).

5.2.3 The establishment of ARMS PCR

CDA 79 A>C, sample analysis was determined in two conditions. The PCR amplification for the condition 1 was created two fragments size as 751 bp for internal control and 194 bp for SNPs A allele, the amplification of condition 2 was created two fragments size as 751 bp for internal control and 105 bp for SNPs C allele.

The PCR amplification of sample showing both fragments of internal control and SNP A allele in condition 1 and only one fragment of internal control in condition 2 was determined as wild type (AA genotype).

The PCR amplification of sample showed only one fragment of internal control in condition 1 and both fragments of internal control and SNPs C allele in condition 2 was determined as variant type (CC genotype)

The PCR amplification of sample showed both fragments of both conditions as heterozygous type. The result of samples amplification in each condition were shown in figure 5.6.

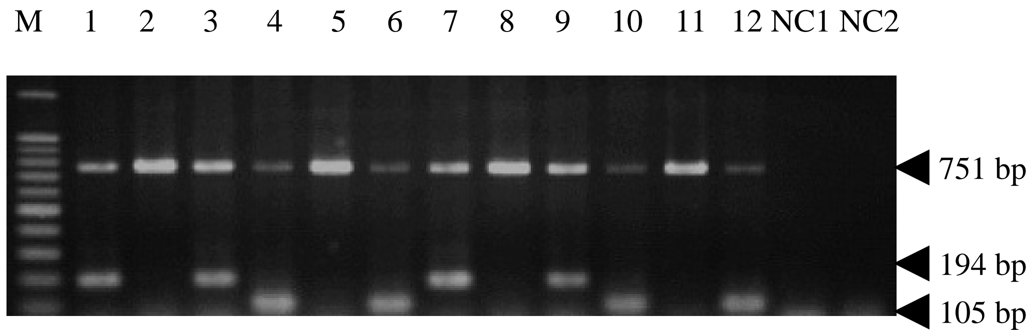


Figure 5.6 The amplification of ARMS PCR.DNA showed ladder (M) was used as molecular marker. To analysis of sample was determined in two conditions, condition 1 and condition 2 respectively. The fragment size show as Lane 1, 194 bp and 751 bp, lane 2, 751 bp, were positive control for wild type (AA genotype), lane 3, 194 bp and 751 bp and lane 4 ,105 bp and 751 bp were positive control for heterozygous (AC genotype). Lane 5, 751 bp and lane 6, 105 bp and 751 bp were positive control for variant. Another lane were unknown sample amplification , lane7 ,194 bp and 751 bp and lane 8, 751 bp for sample 1 was wild type, lane 9,194 bp and 751 bp and lane 10, 105 bp and 751 bp for sample 2 was heterozygous, lane 11, 751 bp and lane 12 ,105 bp and 751 bp for sample 3 was variant. Last of two lanes were negative control for each condition, NC1 was negative control for wild type condition (A allele) and NC2 was negative control for variant condition (C allele).

sequencing Analysis

The amplification of each difference genotype for each SNPs were verified by direct sequencing method using ABI DNA Sequencer 3730 XL genetic analyzer (Perkin, Elmer Applied Biosystems, USA).

For dCK -360 C>G, chromatogram for wild type (CC), heterozygous (CG) and variant type (GG) were shown in figure 5.7-5.9 respectively. For dCK -201 C>T, chromatogram for wild type (CC), heterozygous (CT) and variant type (TT) were shown in figure 5.10-5.12, respectively. For CDA 208 G>A, showed only chromatogram of wild type (GG) in figure 5.13. For CDA 79 C>A were shown

chromatogram for wild type (CC), heterozygous (CA) and variant type (AA) were shown in figure 5.14-5.16 respectively.

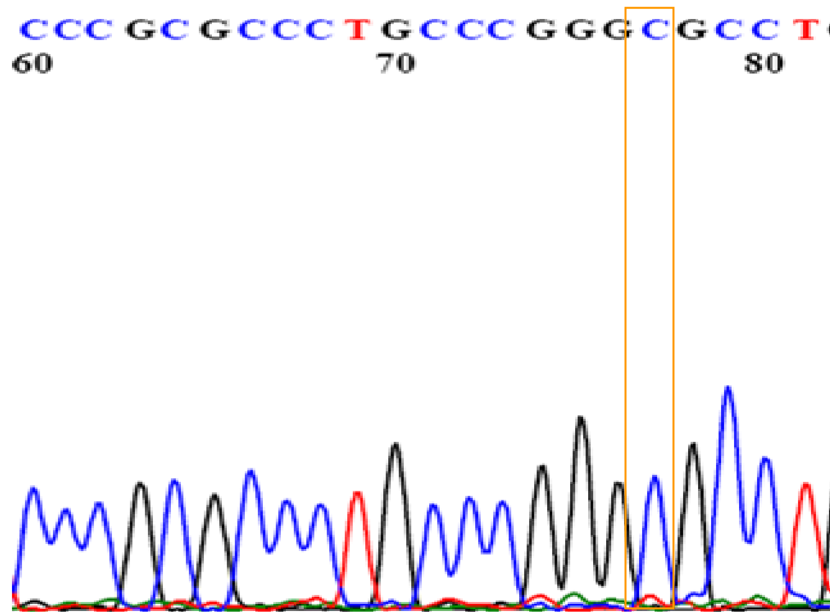


Figure 5.7 Chromatogram of direct sequencing for dCK-360C>G wild type (CC)
In the box showed that dCK-360C>G contain C at nucleotide base at 360 promoter region and had one peak chromatogram.

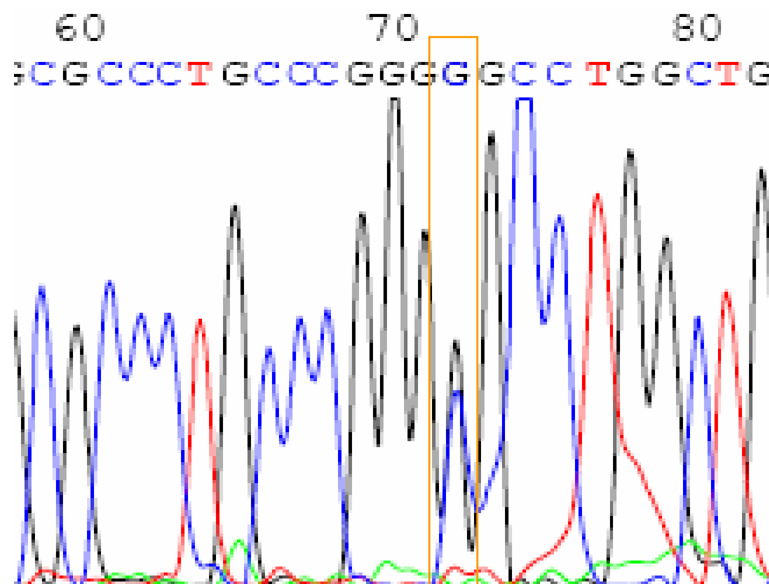


Figure 5.8 Chromatogram of direct sequencing for dCK-360C>G, Heterozygous (CG)
In the box showed that dCK-360C>G had double peak of chromatogram at this position, peak of C and G at 360 promoter region so determined this position as heterozygous.

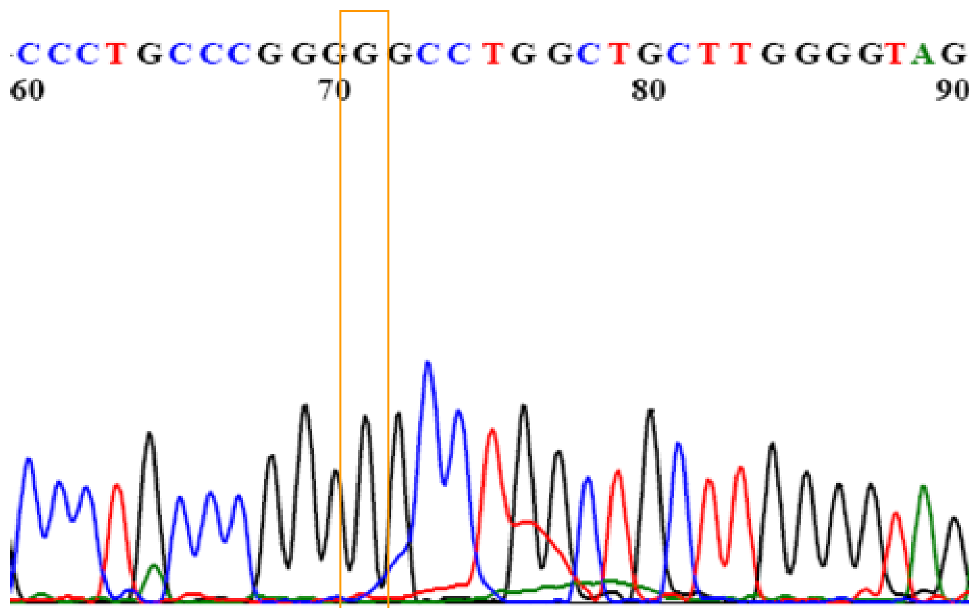


Figure 5.9 Chromatogram of direct sequencing for dCK -360 C>G, Variant (GG)

In the box showed that dCK -360 C>G contain G at nucleotide base at 360 promoter region and had one peak of chromatogram.

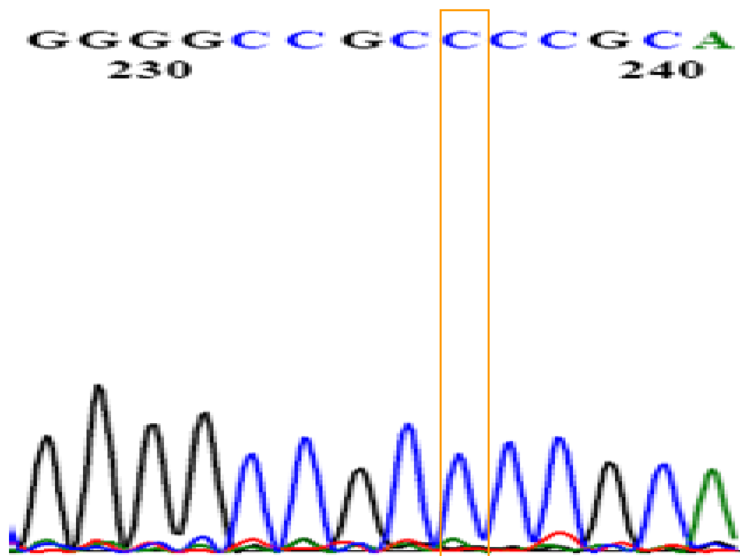


Figure 5.10 Chromatogram of direct sequencing for dCK -201 C>T wild type (CC)

In the box showed that dCK -201 C>T contain C at nucleotide base at 201 promoter region and had one peak chromatogram.

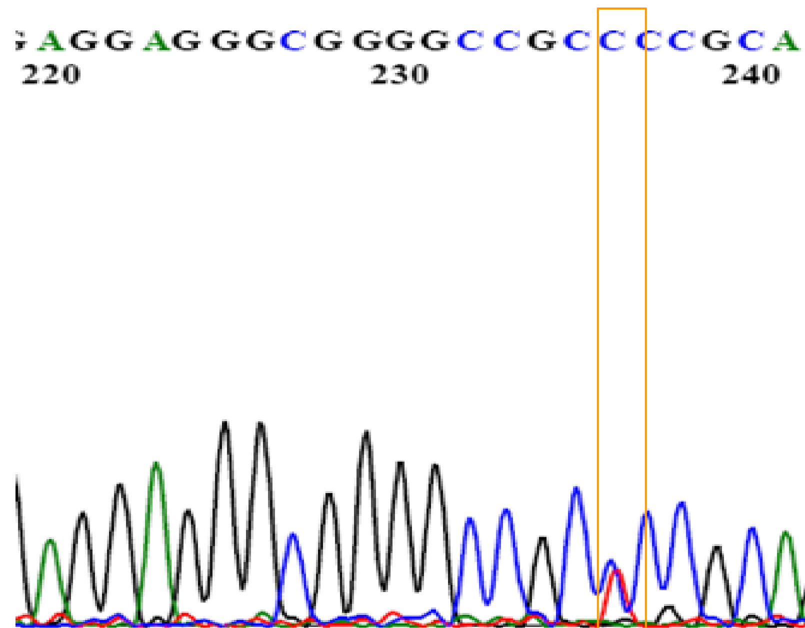


Figure 5.11 Chromatogram of direct sequencing for dCK -201 C>T, Heterozygous (CT)

In the box showed that dCK -201 C>T had double peak of chromatogram at this position, peak of C and T at 201 promoter region so determined this position as heterozygous.

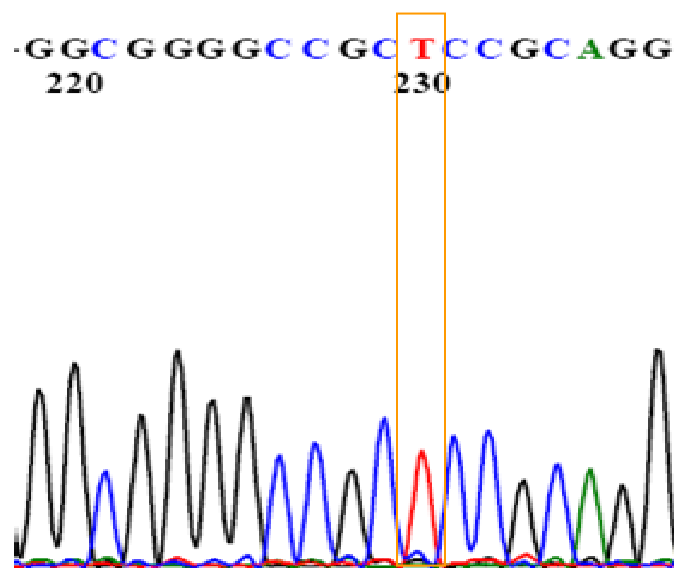


Figure 5.12 Chromatogram of direct sequencing for dCK -201 C>T, Variant (TT)

In the box showed that dCK -201 C>T contain T at nucleotide base at 201 promoter region and had one peak of chromatogram

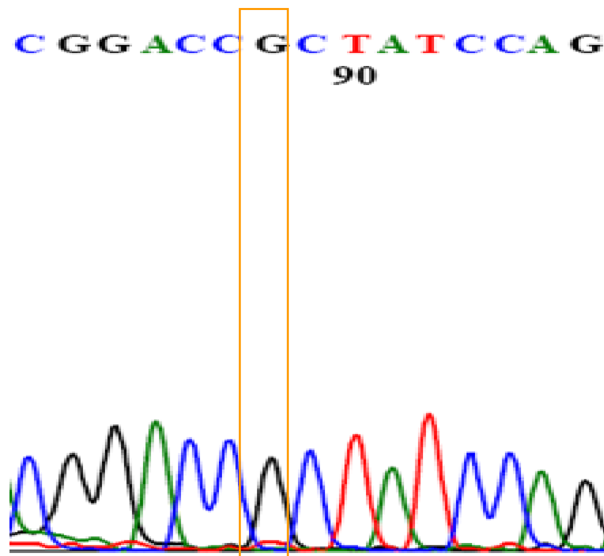


Figure 5.13 Chromatogram of direct sequencing for CDA 208 G>A, wild type (GG)

In the box showed that CDA 208 G>A contain G at nucleotide base at 208 and had one peak chromatogram

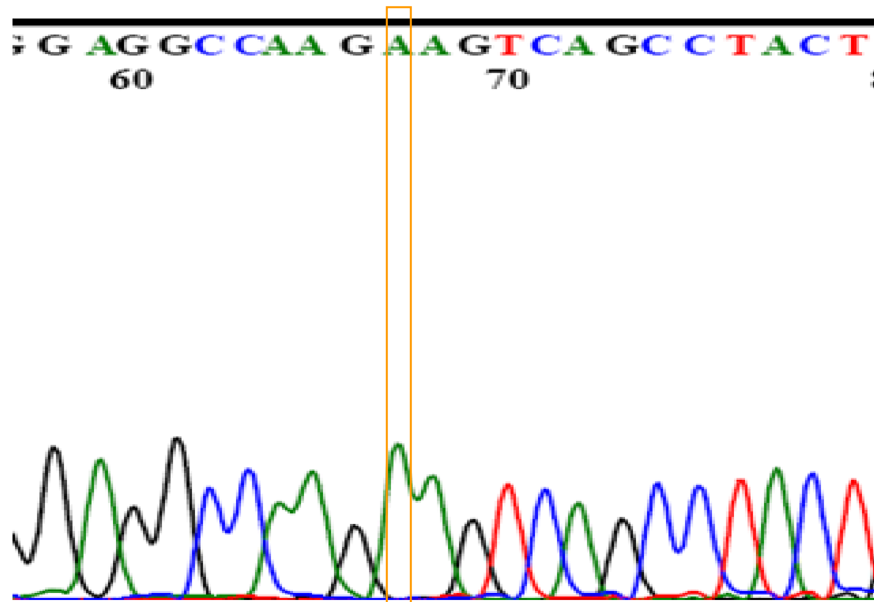


Figure 5.14 Chromatogram of direct sequencing for CDA 79 A>C, wild type (AA)

In the box showed that CDA 79 A>C, contain A at nucleotide base at 79 and had one peak chromatogram

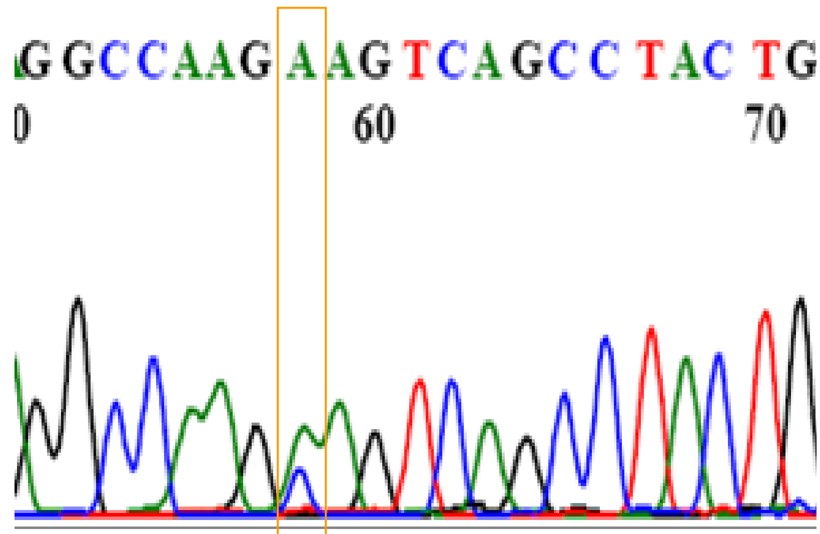


Figure 5.15 Chromatogram of direct sequencing for CDA 79 A>C, Heterozygous

In the box showed that CDA 79 A>C had double peak of chromatogram at this position, peak of A and C at this position so determined this position as heterozygous (AC).

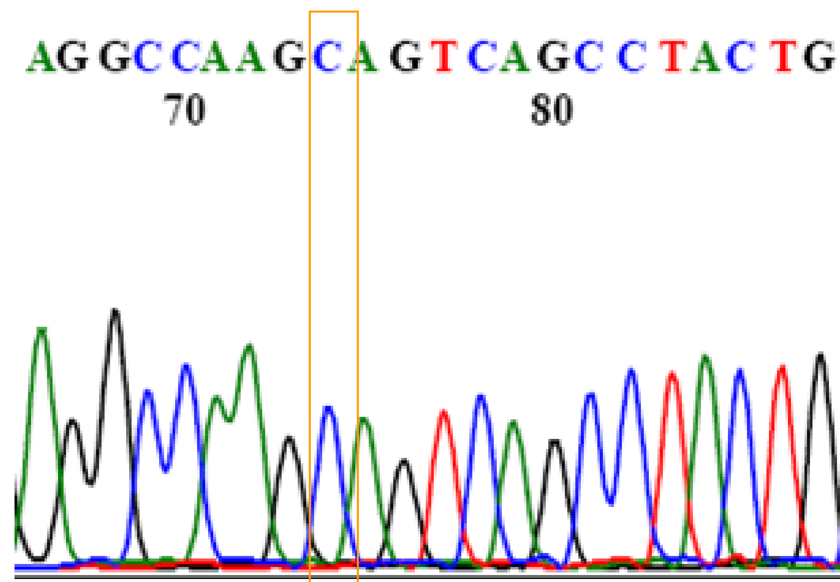


Figure 5.16 Chromatogram of direct sequencing for CDA 79 A>C, Variant

In the box showed that CDA 79 A>C contain C at nucleotide base at this position and had one peak of chromatogram

5.4 Genotyping result

5.4.1 Genotype and alleles frequency of ALL cases and normal control

The distribution of dCK -360 C>G, -201 C>T and CDA 208 G>A, 79 A>C of ALL cases and normal control were shown in Table 5.1 and Table 5.2, respectively. The genotype and alleles frequency of each gene were shown in Table 5.3, Table 5.4 and Table 5.5.

The frequency of dCK -360 C>G and -201 C>T, the genotype -360CC and -201CC (wild type) of ALL cases were 0.72 (72%) whereas the normal control group were 0.70 (70%). The genotype -360 CG and -201 CT (heterozygous) of ALL cases were 0.27 (27%) compare with normal control was 0.26 (26%) and the genotype -360 GG and -201TT (variant) of ALL cases were 0.01 (1%) compare with normal control was 0.04 (4%). The genotype frequency of dCK -360 C>G, -201 C>T in each genotype were not significant difference in ALL cases and normal control (wild type; p=0.84,OR=0.89, heterozygous; p=1.00, OR=0.97, variant; p=0.37, OR=3.88)

The alleles frequency for the major alleles (C allele) of both SNPs (dCK -360 C>G, -201 C>T) of ALL patients and normal control were 0.86 (86%) and 0.83 (83%) respectively, the minor alleles (G or T allele) of both SNPs of ALL patients and normal control were 0.14 (14%) and 0.17 (17%) respectively. There was no statistical difference in the frequency of each allele in these SNPs between ALL cases and normal control (C/C allele; p=0.70, OR=1.26, G/T allele; p=0.70, OR=0.80). The corresponding data both of ALL cases and normal control were shown in Table 5.3.

The frequency of CDA 208 G>A, GG genotype were observed in 100 % of ALL cases and normal control so theirs frequency were 1. Group of genotype and group of allele was no compare, because it had only one genotype and one allele. The corresponding data were shown in Table 5.4 and showed both of ALL cases and normal control.

The frequency of CDA 79 A>C, AA genotype of ALL cases were observed frequency equal 0.87 (87%) compare with normal control was 0.81 (81%), AC genotype of ALL cases was observed frequency equal 0.13 (13%) compare with normal control was 0.18 (18%), CC genotype of ALL cases was no observed but normal control was 0.01 (1%). Group of genotype and group of allele were compared, these were no statistical difference in the frequency of genotype and allele (wild type;

p=0.32 OR=0.62, heterozygous; *p*=0.41 OR= 1.50 and variant *p*= 1, OR-, A allele; *p*=0.43 OR=0.57, C allele; *p*=0.43 OR=1.74) The corresponding data were shown in Table 5.5 and show both of ALL cases and normal control.

Table 5.1 Genotyping of ALL patients

ALL Code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
ALL001	CC	CC	GG	AC
ALL002	CC	CC	GG	AA
ALL003	CC	CC	GG	AA
ALL004	CG	CT	GG	AA
ALL005	CC	CC	GG	AC
ALL006	CG	CT	GG	AA
ALL007	CC	CC	GG	AA
ALL008	CG	CT	GG	AA
ALL009	CC	CC	GG	AA
ALL010	CC	CC	GG	AA
ALL011	CG	CT	GG	AA
ALL012	CG	CT	GG	AC
ALL013	CG	CT	GG	AA
ALL014	CC	CC	GG	AA
ALL015	CC	CC	GG	AA
ALL016	CC	CC	GG	AA
ALL017	CG	CT	GG	AA
ALL018	CC	CC	GG	AA
ALL019	CC	CC	GG	AA
ALL020	CC	CC	GG	AA

ALL Code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
ALL021	CC	CC	GG	AA
ALL022	CC	CC	GG	AA
ALL023	CC	CC	GG	AA
ALL024	CC	CC	GG	AA
ALL025	CC	CC	GG	AA
ALL026	CC	CC	GG	AA
ALL027	CG	CT	GG	AA
ALL028	CC	CC	GG	AA
ALL029	CG	CT	GG	AA
ALL030	CC	CC	GG	AA
ALL031	CG	CT	GG	AA
ALL032	CC	CC	GG	AA
ALL033	CC	CC	GG	AC
ALL034	CC	CC	GG	AA
ALL035	CC	CC	GG	AA
ALL036	CC	CC	GG	AA
ALL037	CG	CT	GG	AA
ALL038	CC	CC	GG	AA
ALL039	CC	CC	GG	AA
ALL040	CC	CC	GG	AA

ALL Code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
ALL041	CC	CC	GG	AA
ALL042	CC	CC	GG	AC
ALL043	CG	CT	GG	AA
ALL044	CC	CC	GG	AA
ALL045	CG	CT	GG	AA
ALL046	CG	CT	GG	AC
ALL047	CC	CC	GG	AA
ALL048	CC	CC	GG	AA
ALL049	CC	CC	GG	AA
ALL050	CC	CC	GG	AA
ALL051	CG	CT	GG	AA
ALL052	CG	CT	GG	AA
ALL053	CC	CC	GG	AA
ALL054	CG	CT	GG	AA
ALL055	CC	CC	GG	AA
ALL056	CG	CT	GG	AA
ALL057	CG	CT	GG	AC
ALL058	CC	CC	GG	AA
ALL059	CC	CC	GG	AC
ALL060	CC	CC	GG	AC

ALL Code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
ALL061	CC	CC	GG	AA
ALL062	CC	CC	GG	AA
ALL063	CG	CT	GG	AA
ALL064	CC	CC	GG	AA
ALL065	CC	CC	GG	AA
ALL066	CC	CC	GG	AA
ALL067	CG	CT	GG	AA
ALL068	CG	CT	GG	AA
ALL069	CC	CC	GG	AA
ALL070	CC	CC	GG	AA
ALL071	CG	CT	GG	AA
ALL072	CC	CC	GG	AA
ALL073	CG	CT	GG	AC
ALL074	CC	CC	GG	AA
ALL075	CC	CC	GG	AC
ALL076	CC	CC	GG	AA
ALL077	CC	CC	GG	AA
ALL078	CC	CC	GG	AA
ALL079	CC	CC	GG	AC
ALL080	CC	CC	GG	AA

ALL Code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
ALL081	CC	CC	GG	AA
ALL082	CC	CC	GG	AA
ALL083	CG	CT	GG	AA
ALL084	CC	CC	GG	AA
ALL085	CC	CC	GG	AA
ALL086	CC	CC	GG	AA
ALL087	CC	CC	GG	AA
ALL088	CC	CC	GG	AA
ALL089	CC	CC	GG	AA
ALL090	CC	CC	GG	AA
ALL091	GG	TT	GG	AA
ALL092	CC	CC	GG	AA
ALL093	CC	CC	GG	AA
ALL094	CC	CC	GG	AA

Table 5.2 Genotyping of Control

Control code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
C001	CC	CC	GG	AA
C002	CC	CC	GG	AC
C003	CC	CC	GG	AC
C004	CC	CC	GG	AA
C005	CG	CT	GG	AA
C006	GG	TT	GG	AA
C007	CC	CC	GG	AA
C008	CC	CC	GG	AA
C009	CC	CC	GG	AA
C010	CC	CC	GG	AA
C011	CG	CT	GG	AA
C012	CC	CC	GG	AA
C013	GG	TT	GG	AA
C014	CC	CC	GG	AA
C015	CG	CT	GG	AA
C016	CC	CC	GG	AA
C017	CC	CC	GG	AA
C018	CC	CC	GG	AA
C019	CC	CC	GG	AA
C020	CC	CC	GG	AC

Control code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
C021	CC	CC	GG	AC
C022	CC	CC	GG	AA
C023	CC	CC	GG	AA
C024	CC	CC	GG	AA
C025	GG	TT	GG	AA
C026	CG	CT	GG	AA
C027	CC	CC	GG	AA
C028	CG	CT	GG	AA
C029	CC	CC	GG	AC
C030	CC	CC	GG	AA
C031	CC	CC	GG	AA
C032	CG	CT	GG	AA
C033	CG	CT	GG	AA
C034	CC	CC	GG	AC
C035	CC	CC	GG	AA
C036	CG	CT	GG	AA
C037	CG	CT	GG	AA
C038	CC	CC	GG	AA
C039	CC	CC	GG	AA
C040	CC	CC	GG	AA

Control code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
C041	CC	CC	GG	AC
C042	CG	CT	GG	AA
C043	CC	CC	GG	AA
C044	CG	CT	GG	AA
C045	CC	CC	GG	AA
C046	CC	CC	GG	AA
C047	CG	CT	GG	AA
C048	CC	CC	GG	AC
C049	CC	CC	GG	AA
C050	CC	CC	GG	AA
C051	CC	CC	GG	AA
C052	CG	CT	GG	AA
C053	CC	CC	GG	AA
C054	CC	CC	GG	AA
C055	CC	CC	GG	AC
C056	CC	CC	GG	AA
C057	CC	CC	GG	AA
C058	CC	CC	GG	AA
C059	CG	CT	GG	AA
C060	CG	CT	GG	CC

Control code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
C061	CC	CC	GG	AA
C062	CC	CC	GG	AA
C063	CC	CC	GG	AA
C064	CG	CT	GG	AC
C065	CG	CT	GG	AA
C066	CC	CC	GG	AA
C067	CC	CC	GG	AA
C068	CC	CC	GG	AA
C069	CC	CC	GG	AA
C070	CG	CT	GG	AC
C071	CC	CC	GG	AA
C072	CC	CC	GG	AA
C073	CC	CC	GG	AA
C074	CG	CT	GG	AA
C075	CC	CC	GG	AA
C076	CG	CT	GG	AA
C077	CG	CT	GG	AA
C078	CG	CT	GG	AC
C079	CC	CC	GG	AA
C080	CC	CC	GG	AA

Control code	Genotyping			
	dCK		CDA	
	-360 C>G	-201 C>T	208 G>A	79 A>C
C081	CC	CC	GG	AA
C082	CC	CC	GG	AC
C083	CG	CT	GG	AA
C084	CC	CC	GG	AA
C085	GG	TT	GG	AA
C086	CG	CT	GG	AA
C087	CC	CC	GG	AC
C088	CC	CC	GG	AA
C089	CC	CC	GG	AC
C090	CG	CT	GG	AA
C091	CG	CT	GG	AA
C092	CC	CC	GG	AA
C093	CC	CC	GG	AC
C094	CC	CC	GG	AA
C095	CC	CC	GG	AA
C096	CC	CC	GG	AA
C097	CC	CC	GG	AC
C098	CC	CC	GG	AA
C099	CC	CC	GG	AC
C100	CC	CC	GG	AA

Table 5.3 Frequency of genotype and alleles of dCK gene (-360 C>G, -201 C>T)

	Genotype frequency			
Genotype	ALL case(n)(%)	control (n) (%)	OR (95%CI)	*p*-value
Wild type	68(72)	70(70)	0.89(0.48-1.66)	0.84
Heterozygous	25(27)	26(26)	0.97(0.51-1.84)	1.00
Variant	1(1)	4(4)	3.88(0.43-35.32)	0.37

	Allele frequency			
Allele	ALL case (%)	Control (%)	OR (95%CI)	*p*-value
C>C	0.86(86)	0.83(83)	1.26(0.58-2.72)	0.70
G>T	0.14(14)	0.17(17)	0.80(0.37-1.72)	0.70

Table 5.4 Frequency of genotype and alleles of CDA gene (208 G>A)

Genotype	ALL case (n)	genotype frequency (%)	control (n)	genotype frequency (%)	Allele frequency G(%)	Allele frequency A(%)
Wild type	94	1(100)	100	1(100)	1(100)	0(0)
Heterozygous	0	0(0)	0	0(0)	-	-
Variant	0	0(0)	0	0(0)	-	-

Table 5.5 Frequency of genotype and alleles of CDA gene (79 A>C)

	Genotype frequency			
genotype	ALL case (n)(%)	Control (n)(%)	OR (95%CI)	*p*-value
Wild type	82(0.87)	81(0.81)	0.62 (0.28-1.36)	0.32
Heterozygous	12(0.13)	18(0.18)	1.50 (0.67-3.31)	0.41
Variant	0(0)	1(0.01)	-	1.00

	Allele frequency			
Allele	ALL case (%)	Control (%)	OR (95%CI)	*p*-value
A	0.94(94)	0.90(90)	0.57(0.20-1.64)	0.43
C	0.06(6)	0.10(10)	1.74(0.60-4.98)	0.43

5.4.2 Association between Genotype, Alleles with AraC toxicity

The criteria of the toxicity in this study was followed criteria of common terminology criteria for adverse events version 4.0 (CTCAE). The toxicity of AraC in this study composed of absolute neutrophil count (<500/mm^3), hemoglobin (Hb) (<8g/dL), Platelet (Plt) (<25,000/mm^3), mucositis, fever, diarrhea, delay of treatment (delay) and febrile neutropenia (FN). The treatments of ALL patients had low dose (remission induction) and high dose (Reinduction II) of AraC. Our study revealed the correlation of each SNPs with AraC toxicity both dose of using drug.

In this study we found that genotype analysis of dCK -360 C>G and-201 C>T were all the same thus they were complete linkage disequilibrium (LD), CDA 208 G>A, we found only GG genotype so we can not find the association of each genotype in this SNPs. The resulting of association between genotype, allele with AraC toxicity were shown at Table 5.6–Table 5.13.

For dCK (-360 C>G, -201 C>T), the correlation between genotype with toxicity of low dose AraC as well as high dose AraC were shown in Table 5.6 and Table 5.7, respectively. The statistically significant difference of SNPs genotypes were shown in using low dose AraC between wild type (CC), and mutant (CG+GG (-360 C/G) or CT+TT(-201 C/T)) with mucositis (p=0.01, 95%CI, OR=7). But in using high dose, there was no statistical significant difference between their genotype and toxicity.

The analysis of allele associated with AraC toxicity for low dose and high dose were show in Table 5.8 and Table 5.9 respectively. For using low dose of AraC, this study show that had statistically significant difference of C allele compared with G allele for-360 C>G or C allele compared with T allele for -201 C>T to be associated with mucositis (p=0.02, 95%CI, OR=3.73). But in using high dose AraC phase were observed not statistical significant between their genotype and toxicity.

For CDA (79 A>C), the association between genotype and toxicity of using low dose and high dose AraC were shown in Table 5.10 and Table 5.11, respectively. Resulting, both dose of using AraC were not statistical significant between their genotype and toxicity. For allele analysis, the SNPs showed no correlation significance between their allele and toxicity (Table 5.12 and Table 5.13).

Table 5.6 Result of association between genotype of dCK -360 C>G, -201 C>T with AraC toxicity (low dose)

Toxicity	Genotype				OR (95%CI)	*p*-value
	CC		CG+GG			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	23 (39.70)	35 (60.30)	11 (50.0)	11 (50.00)	1.52 (0.56-4.08)	0.56
Hb	34 (57.60)	25 (42.40)	15 (68.20)	7 (31.80)	1.57 (0.56-4.43)	0.54
Plt	7 (11.90)	52 (88.10)	1 (4.50)	21 (95.50)	0.35 (0.04-3.05)	0.43
Mucositis	3 (5.10)	56 (94.90)	6 (27.30)	16 (72.70)	7.00 (1.57-31.15)	0.01
Fever	17 (28.80)	42 (71.20)	8 (36.40)	14 (63.60)	1.41 (0.50-3.97)	0.70
Diarhea	5 (8.50)	54 (91.50)	1 (4.50)	21 (95.50)	0.51 (0.05-4.66)	1.00
Delay	18 (30.50)	41 (69.50)	6 (27.30)	16 (72.70)	0.85 (0.28-2.54)	0.99
FN	14 (23.70)	45 (76.30)	8 (36.40)	14 (63.60)	1.83 (0.63-5.27)	0.39

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.7 Result of association between genotype of dCK -360 C>G, -201 C>T with AraC toxicity (High dose)

Toxicity	Genotype				OR (95%CI)	*p*-value
	CC		CG+GG			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	15 (51.70)	14 (48.30)	7 (43.80)	9 (56.20)	0.72 (0.21-2.47)	0.84
Hb	13 (44.80)	16 (55.20)	5 (31.20)	11 (68.80)	0.55 (0.15-2.02)	0.56
Plt	14 (48.30)	15 (51.70)	7 (43.80)	9 (56.20)	0.83 (0.24-2.84)	1.00
Mucositis	2 (6.90)	27 (93.10)	1 (6.20)	15 (93.80)	0.90 (0.07-10.76)	1.00
Fever	9 (31.00)	20 (69.00)	3 (18.80)	13 (81.20)	0.51 (0.11-2.25)	0.49
Diarhea	4 (13.80)	25 (86.20)	2 (12.50)	14 (87.50)	0.89 (0.41-5.50)	1.00
Delay	16 (55.20)	13 (44.80)	12 (75.00)	4 (25.00)	2.43 (0.63-9.38)	0.32
FN	11 (37.90)	18 (62.10)	5 (31.20)	11 (68.80)	0.74 (0.20-2.71)	0.90

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.8 Result of association between allele of dCK -360 C>G, -201 C>T with AraC toxicity (low dose)

Toxicity	Allele				OR (95%CI)	*p*-value
	C		G			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	56 (40.90)	81 (59.10)	12 (52.20)	11 (47.80)	1.57 (0.65-3.82)	0.36
Hb	82 (59.00)	57 (41.00)	16 (69.60)	7 (30.40)	1.58 (0.61-4.11)	0.36
Plt	15 (10.80)	124 (89.20)	1 (4.30)	22 (95.70)	0.37 (0.04-2.99)	0.47
Mucositis	12 (8.60)	127 (91.40)	6 (26.10)	17 (73.90)	3.73 (1.24-11.25)	0.02
Fever	41 (29.50)	98 (70.50)	9 (39.10)	14 (60.90)	1.53 (0.61-3.83)	0.46
Diarhea	11 (7.90)	128 (92.10)	1 (4.30)	22 (95.70)	0.52 (0.06-4.30)	1.00
Delay	42 (30.20)	97 (69.80)	6 (26.10)	17 (73.90)	0.81 (0.30-2.21)	0.80
FN	35 (25.20)	104 (74.80)	9 (39.10)	14 (60.90)	1.91 (0.76-4.79)	0.20

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet ($<25,000/mm^3$), delay; delay of treatment, FN; febrile neutropenia

Table 5.9 Result of association between allele of dCK -360 C>G, -201 C>T with AraC toxicity (High dose)

Toxicity	Allele				OR (95%CI)	*p*-value
	C		G			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	36 (49.30)	37 (50.70)	8 (47.10)	9 (52.90)	0.91 (0.31-2.62)	1.00
Hb	30 (41.10)	43 (58.90)	6 (35.30)	11 (64.70)	0.78 (0.20-2.34)	0.78
Plt	34 (46.60)	39 (53.40)	8 (47.10)	9 (52.90)	1.02 (0.35-2.93)	1.00
Mucositis	4 (5.50)	69 (94.50)	2 (11.80)	15 (88.20)	2.30 (0.38-13.73)	0.31
Fever	20 (27.40)	53 (72.60)	4 (23.50)	13 (76.50)	0.81 (0.23-2.79)	1.00
Diarhea	10 (13.70)	63 (86.30)	2 (11.80)	15 (88.20)	0.84 (0.16-4.24)	1.00
Delay	43 (58.90)	30 (41.10)	13 (76.50)	4 (23.50)	2.26 (0.67-7.63)	0.26
FN	26 (35.60)	47 (64.40)	6 (35.30)	11 (64.70)	0.98 (0.32-2.97)	1.00

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.10 Result of association between genotype of CDA 79 A>C with AraC toxicity (low dose)

Toxicity	Genotype				OR (95%CI)	p-value
	AA		AC			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	29 (41.40)	41 (58.60)	5 (50.00)	5 (50.00)	1.41 (0.37-5.33)	0.73
Hb	42 (59.20)	29 (40.80)	7 (70.00)	3 (30.00)	1.61 (0.38-6.75)	0.73
Plt	7 (9.90)	64 (90.10)	1 (10.00)	9 (90.00)	1.01 (0.11-9.24)	1.00
Mucositis	9 (12.70)	62 (87.30)	0 (0.00)	10 (100.00)	-	0.59
Fever	23 (32.40)	48 (67.60)	2 (20.00)	8 (80.00)	0.52 (0.10-2.65)	0.71
Diarhea	6 (8.50)	65 (91.50)	0 (0.00)	10 (100.00)	-	1.00
Delay	22 (31.00)	49 (69.00)	2 (20.00)	8 (80.00)	0.55 (0.10-2.83)	0.71
FN	20 (28.20)	51 (71.80)	2 (20.00)	8 (80.0.00)	0.63 (0.12-3.26)	0.72

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.11 Result of association between genotype of CDA 79 A>C with AraC toxicity (High dose)

Toxicity	Genotype				OR (95%CI)	p-value
	AA		AC			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	20 (50.00)	20 (50.00)	2 (40.00)	3 (60.00)	0.677 (0.10-4.42)	1.00
Hb	15 (37.50)	25 (62.50)	3 (60.00)	2 (40.00)	2.50 (0.37-16.71)	0.37
Plt	19 (47.50)	21 (52.50)	2 (40.00)	3 (60.00)	0.73 (0.11-4.89)	1.00
Mucositis	2 (5.00)	38 (95.00)	1 (20.00)	4 (80.00)	4.75 (0.34-64.73)	0.30
Fever	11 (27.50)	29 (72.50)	1 (20.00)	4 (80.00)	0.65 (0.06-6.56)	1.00
Diarhea	6 (15.00)	34 (85.00)	0 (0)	5 (100.00)	-	1.00
Delay	24 (60.00)	16 (40.00)	4 (80.00)	1 (20.00)	2.66 (0.27-26.09)	0.63
FN	15 (37.50)	25 (62.50)	1 (20.00)	4 (80.00)	0.41 (0.04-4.08)	0.64

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.12 Result of association between allele of CDA 79 A>C with AraC toxicity (low dose)

Toxicity	Allele				OR (95%CI)	*p*-value
	A		C			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	63 (42.00)	87 (58.00)	5 (50.00)	5 (50.00)	1.38(0.38-4.97)	0.74
Hb	91 (59.90)	61 (40.10)	7 (70.00)	3 (30.00)	1.56 (0.38-6.28)	0.74
Plt	15 (9.90)	137 (90.10)	1 (10.00)	9 (90.00)	1.01 (0.12-8.57)	1.00
Mucositis	18 (11.80)	134 (88.20)	0 (0)	10 (100.00)	-	0.60
Fever	48 (31.60)	104 (68.40)	2 (20.00)	8 (80.00)	0.54 (0.11-2.64)	0.72
Diarhea	12 (7.90)	140 (92.10)	0 (0)	10 (100.00)	-	1.00
Delay	46 (30.30)	106 (69.70)	2 (20.00)	8 (80.00)	0.57 (0.11-2.81)	0.72
FN	42 (26.20)	118 (73.80)	2 (11.10)	16 (88.90)	0.35 (0.07-1.59)	0.24

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.13 Result of association between allele of CDA 79 A>C with AraC toxicity (High dose)

Toxicity	Allele				OR (95%CI)	*p*-value
	A		C			
	Toxic n (%)	No toxic n (%)	Toxic n (%)	No toxic n (%)		
ANC	42 (49.40)	43 (50.60)	2 (40.00)	3 (60.00)	0.68 (0.10-4.29)	1.00
Hb	33 (38.80)	52 (61.20)	3 (60.00)	2 (40.00)	2.36 (0.37-14.90)	0.38
Plt	40 (47.10)	45 (52.90)	2 (40.00)	3 (60.00)	0.75 (0.11-4.71)	1.00
Mucositis	5 (5.90)	80 (94.10)	1 (20.00)	4 (80.00)	4.00 (0.37-42.80)	0.29
Fever	23 (27.10)	62 (72.90)	1 (20.00)	4 (80.00)	0.67 (0.07-6.34)	1.00
Diarhea	12 (14.10)	73 (85.90)	0 (0)	5 (100.00)	-	1.00
Delay	52 (61.20)	33 (38.80)	4 (80.00)	1 (20.00)	2.53 (0.27-23.70)	0.64
FN	31 (36.50)	54 (63.50)	1 (20.00)	4 (80.00)	0.43 (0.04-4.07)	0.65

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

5.4.3 Association between Genotype and MRD

We determine the relationship between genotype and minimal residual disease (MRD) to determine improvement of ALL patients from used AraC. The association between genotype of dCK and CDA genes with MRD were shown in Table 5.14. The correlation of genotype and MRD was determined in two phases of treatment, consolidation therapy and week 7 of reinduction I therapy.

This study observed no significant correlation between MRD and genotype of each gene (dCK and CDA) in consolidation therapy and reinduction I therapy.

Table 5.14 Result of association between Genotype and MRD

Gene	Duration of MRD	Genotype	MRD positive n	MRD Negative n	*p*-value
CDA 79 A>C	Consolidation	AA	11(16.20)	57(83.80)	0.34
		AC	0(0)	9(100.00)	
	Week 7 (Reinduction I)	AA	4(6.90)	54(93.10)	1.00
		AC	0(0)	9(100.00)	
CDA 208 G>A	Consolidation	GG	11(14.30)	66(85.70)	-
	Week 7 (Reinduction I)	GG	4(5.97)	63(94.04)	-
dCK (-360 C>G, -201 C>T)	Consolidation	CC	9(16.10)	47(83.90)	0.71
		CG+GG	2(9.50)	19(90.50)	
	Week 7 (Reinduction I)	CC	2(4.20)	46(95.80)	0.31
		CG+GG	2(10.50)	17(89.50)	

5.4.4 Association between genotype and subtype of ALL

Resulting of genotype were correlated with subtype of ALL (early pre-B cell, pre-B cell and B-cell, T-lineage, mix lineage, lymphoma and undetermined group) in using low and high dose AraC were shown in Table 5.15 and Table 5.16 respectively. The result of these association showed as significant difference between subtype of ALL and genotype of dCK and CDA gene with diarrhea during using high dose of AraC (p=0.01).

Not only that, we were correlated between genotype of each gene with B-lineage and T-lineage and the result showed as no significant both of using dose of AraC (Table 5.17 ,Table 5.18). Whereas the significant difference was found in the correlation between genotype with early pre-B cell and pre-B cell (Table 5.19, Table 5.20) with diarrhea during using high dose AraC (p=0.01 ,95%CI, OR=23).

Table 5.15 The association between Toxicity and subtypes of ALL in low dose phase

Toxicity	early pre-B		Pre-B		B-cell		T- lineage		Mix lineage		Lymphoma		undetermine		*p*-value
	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	
ANC	21	28	5	8	-	-	3	6	2	0	1	3	2	1	0.60
Hb	32	17	6	7	1	0	6	3	1	1	2	2	1	2	0.74
Plt	7	42	1	12	0	1	0	9	0	2	0	4	0	3	0.90
Mucositis	8	41	0	13	0	1	1	8	0	2	0	4	0	3	0.71
Fever	14	35	5	8	0	1	3	6	0	2	2	2	1	2	0.91
diarhea	5	44	0	13	0	1	0	9	0	2	0	4	1	2	0.44
delay	19	30	1	12	0	1	3	6	0	2	1	3	0	3	0.28
FN	13	36	4	9	0	1	3	6	1	1	0	4	1	2	0.83

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.16 The association between Toxicity and subtypes of ALL in high dose phase

Toxicity	early pre-B		Pre-B		B-cell		T-cell		Mix lineage		Lymphoma		undetermine		*p*-value
	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	Toxic	No toxic	
ANC	11	13	3	3	1	0	3	4	1	1	3	1	0	1	0.90
Hb	10	14	0	6	0	1	5	2	0	2	2	2	1	0	0.06
Plt	11	13	2	4	0	1	4	3	0	2	3	1	1	0	0.54
Mucositis	2	22	0	6	0	1	1	6	0	2	0	4	0	1	0.85
Fever	7	17	3	3	1	0	1	6	0	2	0	4	0	1	0.35
diarhea	1	23	3	3	1	0	0	7	1	1	0	4	0	1	0.01
delay	15	9	3	3	1	0	5	2	2	0	1	3	1	0	0.62
FN	7	17	3	3	1	0	2	5	1	1	2	2	0	1	0.69

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.17 The association between Toxicity with B-lineage and T-lineage in low dose phase

Toxicity	T-lineage		B-lineage		OR (95%CI)	*p*-value
	Toxic, n (%)	No toxic, n (%)	Toxic, n (%)	No toxic, n (%)		
ANC	3 (33.30)	6 (66.70)	26 (41.90)	36 (58.10)	1.44 (0.33-6.31)	0.72
Hb	6 (66.70)	3 (33.30)	39 (61.90)	24 (38.10)	0.81 (0.18-3.55)	1.00
Plt	0 (0)	9 (100.00)	8 (12.70)	55 (87.30)	-	0.58
Mucositis	1 (11.10)	8 (88.90)	8 (12.70)	55 (87.30)	1.16 (0.12-10.57)	1.00
Fever	3 (33.30)	6 (66.70)	19 (30.20)	44 (69.80)	0.86 (0.19-3.81)	1.00
diarhea	0 (0)	9 (100.00)	5 (7.90)	58 (92.10)	-	1.00
delay	3 (33.30)	6 (66.70)	20 (31.70)	43 (68.30)	0.93 (0.21-4.10)	1.00
FN	3 (33.30)	6 (66.70)	17 (27.00)	46 (73.00)	0.73 (0.16-3.29)	0.70

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.18 The association between Toxicity with B-lineage and T-lineage in high dose phase

Toxicity	T-lineage		B-lineage			
	Toxic, n (%)	No toxic, n (%)	Toxic, n (%)	No toxic, n (%)	OR (95%CI)	*p*-value
ANC	3 (42.90)	4 (57.10)	15 (48.40)	16 (51.60)	1.25 (0.23-6.53)	1.00
Hb	5 (71.40)	2 (28.60)	10 (32.30)	21 (67.70)	0.19 (0.03-1.15)	0.08
Plt	4 (57.10)	3 (42.90)	13 (41.90)	18 (58.10)	0.54 (0.10-2.84)	0.67
Mucositis	1 (14.30)	6 (85.70)	2 (6.50)	29 (93.50)	0.41 (0.03-5.33)	0.46
Fever	1 (14.30)	6 (85.70)	11 (35.50)	20 (64.50)	3.30 (0.35-31.03)	0.39
diarhea	0 (0)	7 (100.00)	5 (16.10)	26 (83.90)	-	0.56
delay	5 (71.40)	2 (28.60)	19 (61.30)	12 (38.70)	0.63 (0.10-3.80)	1.00
FN	2 (28.60)	5 (71.40)	11 (35.50)	20 (64.50)	1.37 (0.22-8.29)	1.00

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Platelet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

Table 5.19 The association between Toxicity with early pre B cell and Pre B cell in low dose phase

Toxicity	early pre-B		Pre-B			
	Toxic, n(%)	No toxic, n(%)	Toxic, n(%)	No toxic,n(%)	OR (95%CI)	*p*-value
ANC	21 (42.90)	28 (57.10)	5 (38.50)	8 (61.50)	0.83 (0.23-2.91)	1.00
Hb	32 (65.30)	17 (34.70)	6 (46.20)	7 (53.80)	0.45 (0.13-1.57)	0.22
Plt	7 (14.30)	42 (85.70)	1 (7.70)	12 (92.30)	0.50 (0.05-4.47)	1.00
Mucositis	8 (16.30)	41 (83.70)	0 (0)	13 (100.00)	-	0.18
Fever	14 (28.60)	35 (71.40)	5 (38.50)	8 (61.50)	1.56 (0.43-5.60)	0.51
diarhea	5 (10.20)	44 (89.80)	0 (0)	13 (100.00)	-	0.57
delay	19 (38.80)	30 (61.20)	1 (7.70)	12 (92.30)	0.13 (0.01-1.09)	0.05
FN	13 (26.50)	36 (73.50)	4 (30.80)	9 (69.20)	1.23 (0.32-4.68)	0.73

ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Pletlet (<25,000/mm^3), delay of treatment, FN; febrile neutropenia

Table 5.20 The association between Toxicity with early pre B cell and Pre B cell in high dose phase

Toxicity	early pre-B		Pre-B		OR (95%CI)	*p*-value
	Toxic, n (%)	No toxic, n (%)	Toxic, n (%)	No toxic, n (%)		
ANC	11 (45.80)	13 (54.20)	3 (50.00)	3 (50.00)	1.18 (0.19-7.08)	1.00
Hb	10 (41.70)	14 (58.30)	0 (0)	6 (100.00)	-	0.07
Plt1	11 (45.80)	13 (54.20)	2 (33.30)	4 (66.70)	0.59 (0.09-3.86)	0.67
Mucositis	2 (8.30)	22 (91.70)	0 (0)	6 (100.00)	-	-
Fever	7 (29.20)	17 (70.80)	3 (50.00)	3 (50.00)	2.42 (0.39-15.08)	0.37
diarhea	1 (4.20)	23 (95.80)	3 (50.00)	3 (50.00)	23.00 (1.77-298.45)	0.01
delay	15 (62.50)	9 (37.50)	3 (50.00)	3 (50.00)	0.60 (0.09-3.63)	0.66
FN	7 (29.20)	17 (70.80)	3 (50.00)	3 (50.00)	2.42 (0.39-15.08)	0.37

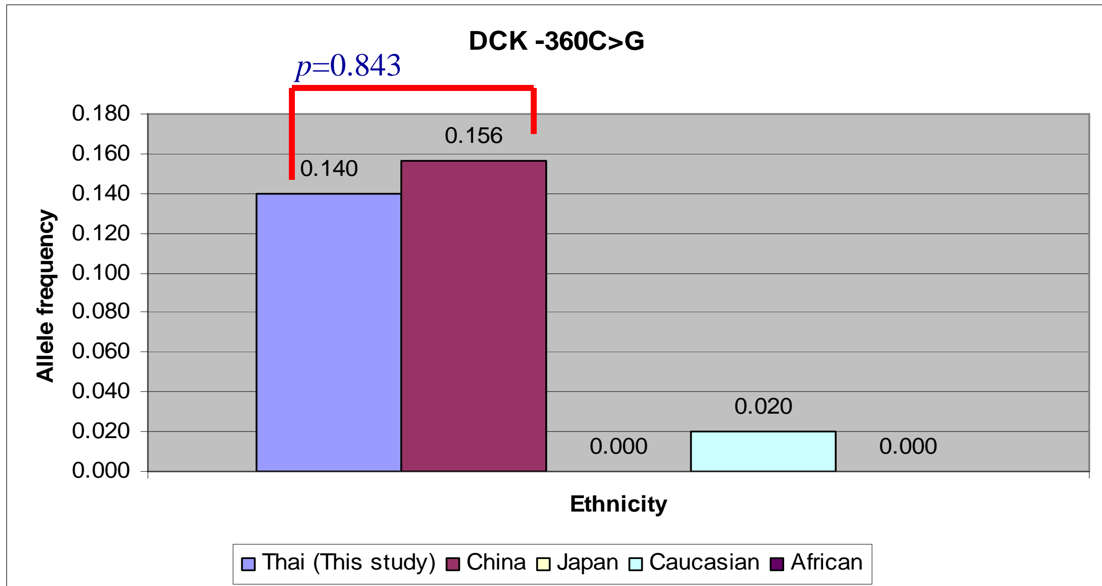
ANC; Absolute neutrophil count, Hb; Hemoglobin, Plt; Pletlet (<25,000/mm^3), delay; delay of treatment, FN; febrile neutropenia

5.4.5 Associated of polymorphism of dCK and CDA with other ethnicity

For the frequency of dCK and CDA genes in this study (14%) and other report previously of China, Japan, Caucasians and Africans population were shown in Table 5.21. The allelic frequency of dCK (-360 C>G, -201 C>T) were 0.156 (15.6%) and 0.002 (2%) for China and Caucasians, respectively. The allelic frequency of CDA 79A>C were 0.201-0.207 (20.1-20.7%), 0.298-0.36 (29.8-36%) and 0.04-0.108 (4-10.8%) for Japan, Caucasians and Africans population, respectively. The allelic frequency of CDA 208 G>A were 0.037-0.043 (3.7-4.3%) and 0.13 (13%) for Japan and Africans population, and this SNPs not had seen in Caucasians population.

For association between dCK, CDA in each population with Thai population in this study found that dCK (-360 C>G, -201 C>T) not significantly difference in China and Thai population (p=0.843, 95%CI). The association of CDA 79 C>A in Thai population with each ethnicity were significantly difference in Thai compared with Japan population (p=0.006-0.009,95%CI) and Thai compared with Caucasians population too (p=0.001-<0.001, 95%CI) but not significantly difference in Thai compared with Africans population (p=0.518, 95%CI). The associated of CDA 208 G>A in Thai population with each ethnicity were not significantly difference in Thai and Japan population (p=0.05, 95%CI) but it was significantly difference between Thai and Africans population (p=<0.001, 95%CI).

Figure 5.21 Graph of SNPs Frequenct of the dCK gene in difference population



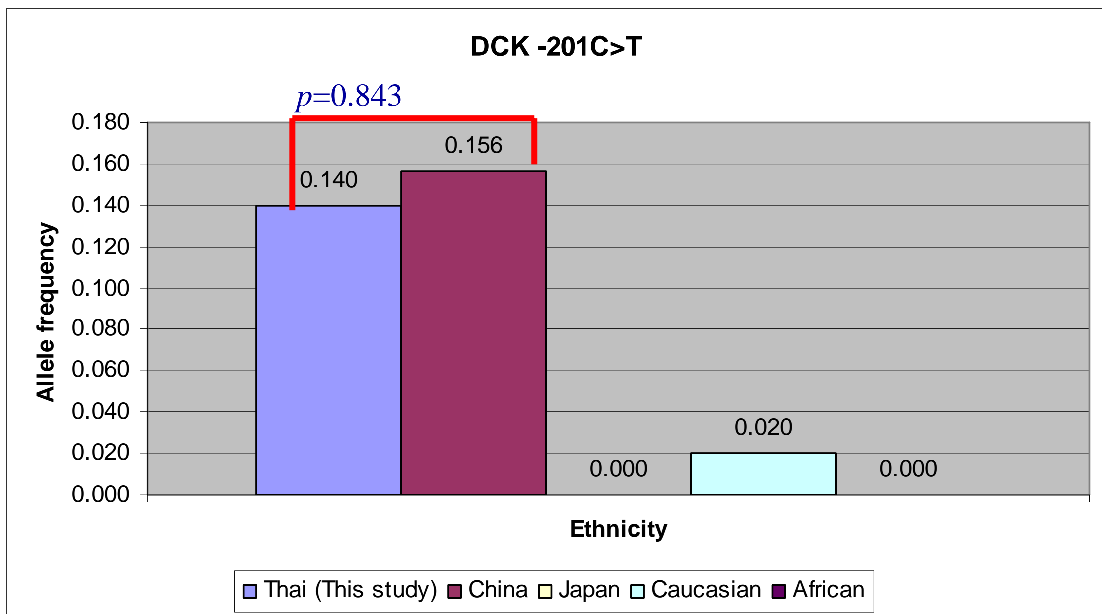
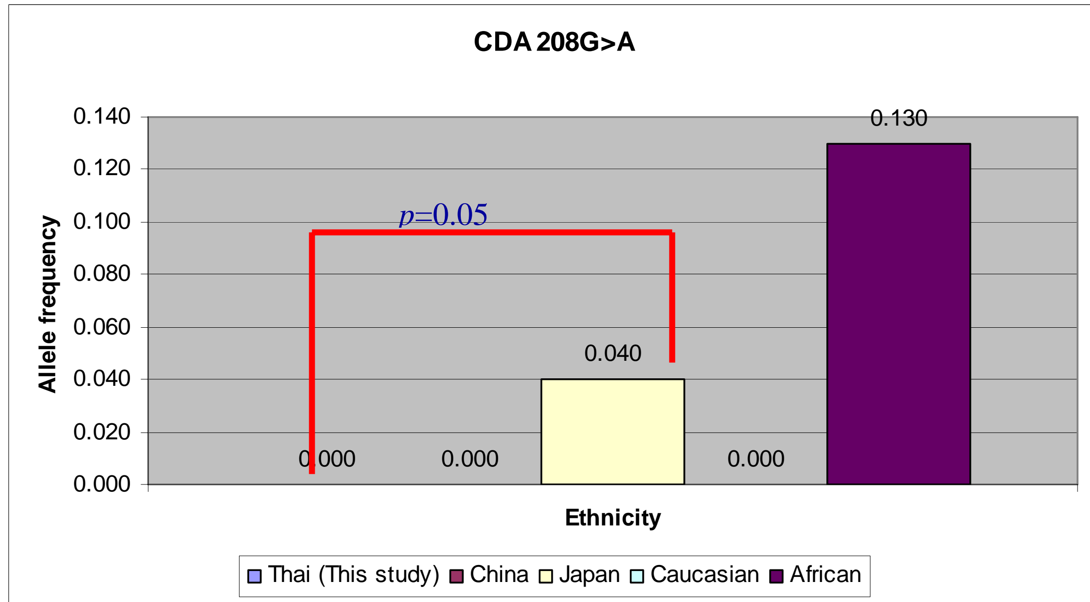
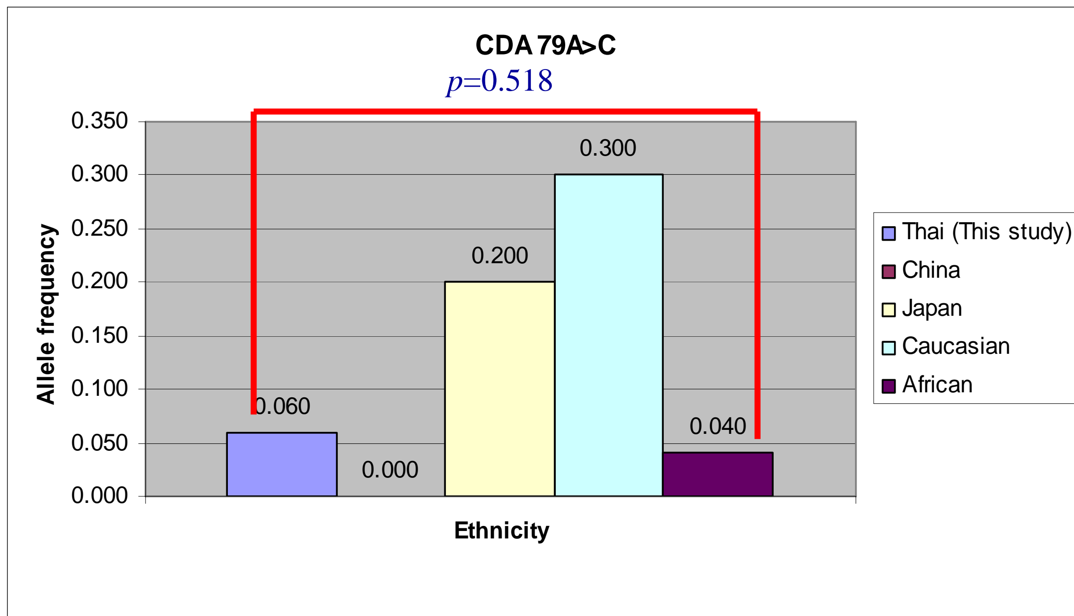


Figure 5.21 Graph of SNPs Frequenct of the CDA gene in difference population





5.5 The result of quantitative real-time PCR

5.5.1 mRNA Expression

The result of mRNA expression level of dCK and CDA normalized with beta actin were shown in Table 5.22.

The normalization were the relative expression of target gene (dCK, CDA) with endogenous housekeeping gene (internal control or reference gene) as beta-actin in the same cDNA template.

Table 5.21 The result of mRNA expression level of dCK and CDA normalized with beta actin

ALL code	mRNA expression		Genotype			
	DCK	*CDA*	dCK -360 C>G	dCK -201C>T	CDA 79A>C	CDA 208 G>A
AL001	0.04142	0.02409	CC	CC	AA	GG
AL002	0.11772	0.12351	CG	CT	AA	GG
AL003	0.07666	0.189	CC	CC	AA	GG
AL004	0.10621	0.07391	CG	CT	AA	GG
AL005	0.46984	0.36391	CC	CC	AA	GG
AL006	0.24305	0.13153	CC	CC	AA	GG
AL007	0.47827	0.14246	CC	CC	AA	GG
AL008	0.05604	0.17453	CC	CC	AA	GG
AL009	1.13788	0.01371	CC	CC	AA	GG
AL010	0.00243	0.05723	CC	CC	AA	GG
AL011	0.19035	0.01741	CG	CT	AA	GG
AL012	0.10147	0.04213	CC	CC	AA	GG
AL013	0.64696	0.08863	CG	CT	AA	GG
AL014	0.71696	2.30773	CC	CC	AA	GG
AL015	0.43009	0.78222	CG	CT	AA	GG
AL016	0.40787	0.03451	CC	CC	AA	GG
AL017	0.26965	0.53809	CC	CC	AC	GG
AL018	0.07929	0.3633	CC	CC	AA	GG
AL019	1.39207	0.78039	CC	CC	AA	GG
AL020	0.56699	0.41663	CC	CC	AA	GG

	mRNA expression		Genotype			
ALL code	*DCK*	*CDA*	dCK -360 C>G	dCK -201 C>T	CDA 79 A>C	CDA 208G>A
AL021	0.9084	0.84192	CG	CT	AA	GG
AL022	0.84099	0.0308	CC	CC	AA	GG
AL023	0.90093	0.95894	CC	CC	AA	GG
AL024	0.20596	0.00167	CC	CC	AA	GG
AL025	0.30907	2.22952	CG	CT	AA	GG
AL026	2.962	0.54419	CG	CT	AA	GG
AL027	1.35114	0.43523	CG	CT	AA	GG
AL028	0.97336	0.01388	CC	CC	AA	GG
AL029	0.7616	0.00953	CC	CC	AC	GG
AL030	0.71287	0.73821	CC	CC	AA	GG
AL031	0.60148	0.00007	CG	CT	AA	GG
AL032	1.77212	0.01264	CG	CT	AA	GG
AL033	1.0543	0.01717	CC	CC	AA	GG
AL034	0.40001	0.17261	CG	CT	AA	GG
AL035	2.2825	0.35549	CG	CT	AA	GG
AL036	6.03068	0.04451	CG	CT	AC	GG
AL037	0.5303	0.06454	CC	CC	AA	GG
AL038	1.84477	0.0134	CC	CC	AC	GG
AL039	0.23075	0.38494	CC	CC	AA	GG
AL040	3.7698	0.18346	CC	CC	AA	GG
AL041	0.42962	2.24498	CC	CC	AA	GG
AL042	0.29894	1.9832	CC	CC	AA	GG
AL043	24.2174	0.0824	CG	CT	AA	GG
AL044	4.75335	0.05763	CC	CC	AA	GG

5.5.2 Association between mRNA expression and Genotype

The resulting of these association showed that no significant difference between mRNA expression and genotype of dCK -360 C>G, -201 C>T (95% CI, *p*=0.12) and CDA 79 A>C (95% CI, *p*=0.17) were shown at Table 5.23. But we did not associated 208 G>A genotype with *CDA* expression because of the genotype of its was constants (GG genotype only).

Table 5.22 The relative mRNA level of dCK and CDA expression

Gene	Polymorphism	Genotype	mRNA expression (Median)	*p*-value
CDA	79 A>C	AA (n=40)	0.15753 (0.00007-2.30773)	0.18
		AC (n=4)	0.02895 (0.00953-0.53809)	
		CC (n=0)		
	208 G>A	GG (n=44)	0.13699 (0.00007-2.30773)	-
		GA (n=0)		
		AA (n=0)		
dCK	-201 C>T	CC (n=29)	0.46984 (0.00243-4.75335)	0.12
		CT (n=15)	0.71695 (0.10621-24.21738)	
		TT (n=0)		
	-360 C>G	CC (n=29)	0.46984 (0.00243-4.75335)	0.12
		CG (n=15)	0.71695 (0.10621-24.21738)	
		GG (n=0)		

5.5.3 Association between mRNA expression and MRD

The resulting showed as the association between mRNA expression and MRD of CDA and dCK in two phases of treatment. The CDA at consolidation phase showed not significant difference between mRNA expression and MRD (*p*=0.89) but showed significantly difference at week 7 of reinduction I phase (*p*=0.04). The dCK showed not significant difference in both phases of treatment, consolidation and reinduction I phase, respectively (*p*=0.87, *p*=0.39). The data of the association were shown at Table 5.24.

Table 5.23 The association between mRNA expression and MRD

Gene	Duration of MRD	mRNA Expression,(Median)		*p*-value
		MRD positive	MRD Negative	
CDA	Consolidation*	0.1629 (0.0137-2.2295)	0.1061 (0.0001-2.3077)	0.89
	Week 7** (Reinduction I)	0.0069 (0.0001-0.0137)	0.1315 (0.0017-2.3077)	0.04
dCK	Consolidation*	0.4431 (0.0137-2.2295)	0.5842 (0.0024-24.2174)	0.87
	Week 7** (Reinduction I)	0.8697 (0.6015-1.1379)	0.04698 (0.0024-24.2174)	0.39

* The consolidation phase, MRD positive samples were 8 cases and the samples of MRD negative were 32 cases.
** The Reinduction I phase (week 7), MRD positive samples were 2 cases and MRD negative samples were 33 cases.

CHAPTER VI
DISCUSSION

AraC resistance is one of the major problems of acute leukemia treatment. The genes that encodes drug metabolizing enzymes, transporters or drug targets can influence the efficacy and toxicity of chemotherapy [92, 93] Deoxycytidine kinase (dCK) is a rate-limiting enzyme of the intracellular phosphorylation of nucleoside anticancer drugs including cytarabine (AraC), converted to active metabolite (AraCTP) by dCK gene. CDA is an enzyme involved in the metabolism of AraC , which degraded by CDA, forms the inactive product (AraU). There are various mechanisms involved in cellular AraC (cytarabine) resistance such as dCK deficiency and/or CDA increase in its pathogenesis [94-100], low activity of dCK [11], the structure of gene mutation in dCK [75] and the balance of activating enzymes (dCK) and degrading enzymes (CDA), thus is crucial in determining the AraC cytotoxicity [19,65].

The alleic frequency of the promoter polymorphism of dCK -360 C>G and -201 C>T of Thai poppulation (14%) and other ethnicity showed the slightly significant difference of these genotypes in Caucasians population (p=0.003) whereas there was no significant difference between Thai population and Asian population (China) (p=0.843)[7,18]. High frequency of dCK might predispose Thai and Asian populations associated toxicity to Ara- C. However, the allelic frequency of the promoter polymorphism dCK -360 C>G and dCK -201 C>T showed no significant difference between childhood ALL patients and normal control group. This data indicated that genetic variation of this gene not differently in ALL patients and normal controls.

Our genotypic study of two SNPs dCK (-360 C>G and -201 C>T) in acute lymphoblastic leukemia (ALL) and control group, showed three different genotypes as wild type heterozygous and variant in the percentage of 72, 27, 1 and 70, 26, 4,

respectively. The study by Shi et al confirmed [7] that two SNP of dCK -360 C>G and -201 C>T were in complete linkage disequilibrium(LD).

The association between the toxicity of AraC in childhood ALL patients with low- or high-dose treatment and the DNA sequence polymorphism of dCK demonstrated that only low-dose of AraC treatment. The mutant variation of dCK, heterozygous (-360 CG/-201 CT) and variant (-360 GG/ -201 TT) showed increased risk of mucositis after a low dose of AraC. This might be due to the influence of the genetic factors involved with in toxicity. The accumulation of AraC in cell was occurred drug toxicity in ALL patients during two phase of using AraC. The AraC toxicity showed significant only low dose phase but high dose phase did not showed significantly because in this phase the patients was received growth factor for this treatment.

Our study showed no correlation between the genetic variation of dCK and its mRNA expression. This was contrasted to the previous study reported showing variation in dCK mRNA expression in 10 childhood ALL cases. In AML patients the genetic variation was also influenced by dCK mRNA expression [68] and clinical outcome [10, 101]. The level of mRNA expression in AML patients with -360 CG/ -201 CT and -360 GG/ -201 TT was higher than in those with -360 CC/-201 CC [7]. The decreased dCK mRNA expression was correlated with decreased dCK activity and conferred to AraC resistance in AML leukemic cell lines [72, 102-105], the leukemic cell line might function ship in *vivo* expression but not in *vitro*.

For CDA gene, we explored two non-synonymous SNPs: CDA 79 A>C as the recombinant protein carrying the missense variant L27Q and CDA 208 G>A the threonine variant of A70T.

The allelic frequency of CDA 79 A>C in Thai population and other ethnicity showed significant difference between Thai population compared to Asian (Japan) [18, 19, 22] and Caucasians population [18, 20, 21] but not in Africans population [18, 20, 21]. However, the identified polymorphism of CDA 79 A>C, showed no significant difference between allelic frequency of childhood ALL patients and control group. This data indicated that genetic variation of this gene not differently in ALL patients and normal controls.

The allelic frequency of CDA 208 G>A was observed only in wild type, GG genotype, both of childhood ALL patients and of normal control group, indicating that CDA 208 G>A had a very low or lack of variant polymorphism of this SNPs in the Thai population. No significant difference in the allelic frequency in Thai population and Asian population (Japan) [18, 19, 22] was found but the significant difference was showed between Thai population and Africans population [18, 20, 21].

In our genotypic study of CDA 79 A>C, ALL patients showed two genotypes as wild type and heterozygous whereas the control group showed three genotypes as wild type, heterozygous and variant in the percentage of 87, 13, 0 and 81,18,1, respectively.

The genotypic study showed no association between the toxicity of AraC chemotherapy in childhood ALL patients in low- and high- dose of AraC treatment. This report did not direct measurement of CDA activity that in another study was found to reveal the reducing activity of variant C allele genotype compared to wild type A allele genotype [21, 106]. The CC genotype (variant) was at an increased risk of postinduction treatment related mortality (TRM) with high dose AraC base therapy at postinduction outcome [107], Mahlknecht *et al* [48] reported AC genotype (heterozygous) had liver toxicity more than AA genotype (wild type), so the mechanism of CDA 79 A>C activity by direct measurement needs further investigation. This study we study *in vitro* CDA activity, we did not seen differently. The CDA might be had many factor to involve it’s activity so to understand of CDA activity we might to test *in vivo* too.

For CDA 208 G>A, in this study we did not observe different genotypes of this SNPs, so we did not associate it with other factors. Yonemori *et al* [24] reported that a patient with homozygous 208 A alleles of CDA showed severe adverse reactions and Sugiyama *et al* [22] found association between genetic polymorphism and toxicity of gemcitabine in CDA 208 G>A

Our study also showed no association between CDA mRNA expression in each genetic variation as well as mRNA expression with AraC toxicity. In vitro study, the resistant cell to gemcitabine, as cytarabine analogue, had CDA over expression [54] whereas the cell with lower CDA expression had longer overall survival [108].

No relationship between each genotype in two phases of treatment, consolidation and reinduction II phase and minimal residual disease (MRD), this might be the low of samples size. The correlation between MRD and mRNA expression was not significantly different in dCK, but showed significant difference in CDA. The association between MRD and mRNA expression in re-induction I of treatment showed statistic significant difference (p=0.04). The MRD positive patients were less mRNA expression than group of MRD negative, this data indicated mRNA expression of CDA revealed with childhood ALL patients improvement.

According to high dose of AraC drug toxicity with the group of diseases including ALL subtype, B and T-lineage, mix lineage, relapse ALL, Lymphoma and unknown group revealed the association with diarrhea .However, no correlation was shown between B and T-lineage with AraC toxicity. Only early pre-B ALL and pre-B ALL subtype with high dose of AraC toxicity, associated with diarrhea but the interval range of odd ratio was wildly, because of low of samples size.

To study of drug toxicity have to use peripheral blood sample from who complete remission because we want to study on germ line of patients. The response of AraC toxicity often to study in germ line. The responsive study of treatment have to use bone marrow sample because it investigate in cancer cell.

This study, we have to compare genotype of dCK and CDA in two group of sample (germ line and leukemic cell). The comparison in each SNPs of two gene were all the same, this data indicated that genetic variation did not difference in two samples groups.

In summary, our study was the first reported of interethnic gene that the frequency of allelic variant of dCK of Thai was similar to China population but differed from Caucasians population. The ethnic differences might indicated the outcome in response to AraC treatment in the two populations [109]. Only the dCK genetic alterations correlated with toxicity of low-dose AraC in childhood ALL. According to the risk to toxicity, the variant polymorphism of dCK -360 CG/ -201 CT or -360 GG/ -201 TT were more at risk to mucositis than wild type polymorphism of dCK -360 CC/ -201 CC. CDA did not associate with toxicity of AraC in both of treatments. The mRNA expression of both genes in each genotype showed no significant difference in each genotype.

The association between MRD and genotype or mRNA expression showed significance only during CDA mRNA expression. However, the childhood ALL had multifactor mechanisms to be concerned about. Future studies of these various mechanisms could help individualize chemotherapy and therefore potentially improve the condition of persons with ALL.

CHAPTER VII
CONCLUSION

Our study showed that there was no significant difference in the alleic frequency of dCK and CDA genes between normal control and ALL case in Thai population. This study was the first report allelic variant frequency of dCK and CDA genes in Thai population. The study was investigated in association between alleic genotype and AraC toxicity, in ALL patients who had genotype as mutant of alleic dCK as heterozygous and variant genotype (-360 CG/-201 CT, -360 GG/ -201 TT) were increased risk of AraC related mucositis after low dose AraC. We did not found significant association of AraC toxicity in alleic genotype of CDA, might be this gene has many factor to involve in it's activity. The dCK and CDA genetic variations did not alter their mRNA expression. About MRD and genotype, MRD and mRNA expression showed significant only CDA mRNA expression. In summary, this study indicated that dCK and CDA were the importance enzymes in AraC metabolite.

REFERENCES

1 Pui C-H, Robison LL, Look AT. Acute lymphoblastic leukaemia. The Lancet.371(9617):1030-43.

2 Stam RW, Hubeek I, den Boer ML, Buijs-Gladdines JG, Creutzig U, Kaspers GJ, *et al*. MLL gene rearrangements have no direct impact on Ara-C sensitivity in infant acute lymphoblastic leukemia and childhood M4/M5 acute myeloid leukemia. Leukemia. 2006 Jan;20(1):179-82.

3 Cai J, Damaraju VL, Groulx N, Mowles D, Peng Y, Robins MJ, *et al*. Two distinct molecular mechanisms underlying cytarabine resistance in human leukemic cells. Cancer Res. 2008 Apr 1;68(7):2349-57.

4 Momparler RL. Kinetic and template studies with 1-β-D-arabinofuranoxyl cytosine-5’-triphosphate and mammalian deoxyribonucleic and polymerase. Mol Pharmacol. 1972;8:362-70.

5 Kufe DW, Major PP, Egan EM, Beardsley GP. Correlation of cytotoxicity with incorporation of ara-C into DNA. J Biol Chem. 1980 Oct 10;/255(19):8997-900.

6 Mansson E, Liliemark E, Soderhall S, Gustafsson G, Eriksson S, Albertioni F. Real-time quantitative PCR assays for deoxycytidine kinase, deoxyguanosine kinase and 5'-nucleotidase mRNA measurement in cell lines and in patients with leukemia. Leukemia. 2002 Mar;16(3):386-92.

7 Shi JY, Shi ZZ, Zhang SJ, Zhu YM, Gu BW, Li G, *et al*. Association between single nucleotide polymorphisms in deoxycytidine kinase and treatment response among acute myeloid leukaemia patients. Pharmacogenetics. 2004 Nov;14(11):759-68.

8 Joerger M, Bosch TM, Doodeman VD, Beijnen JH, Smits PH, Schellens JH. Novel deoxycytidine kinase gene polymorphisms: a population screening study in Caucasian healthy volunteers.Eur J Clin Pharmacol.2006Aug;62(8):681-4.

9 Kakihara T, Fukuda T, Tanaka A, Emura I, Kishi K, Asami K, *et al*. Expression of deoxycytidine kinase (dCK) gene in leukemic cells in childhood: decreased expression of dCK gene in relapsed leukemia. Leuk Lymphoma. 1998 Oct;31(3-4):405-9.

10 Stammler G, Zintl F, Sauerbrey A, Volm M. Deoxycytidine kinase mRNA expression in childhood acute lymphoblastic leukemia. Anticancer Drugs. 1997 Jun;8(5):517-21.

11 Tattersall MH, Ganeshaguru K, Hoffbrand AV. Mechanisms of resistance of human acute leukaemia cells to cytosine arabinoside. Br J Haematol. 1974 May;27(1):39-46.

12 Colly LP, Peters WG, Richel D, Arentsen-Honders MW, Starrenburg CW, Willemze R. Deoxycytidine kinase and deoxycytidine deaminase values correspond closely to clinical response to cytosine arabinoside remission induction therapy in patients with acute myelogenous leukemia. Semin Oncol. 1987 Jun;14(2 Suppl 1):257-61.

13 Kawasaki H, Carrera CJ, Piro LD, Saven A, Kipps TJ, Carson DA. Relationship of deoxycytidine kinase and cytoplasmic 5'-nucleotidase to the chemotherapeutic efficacy of 2-chlorodeoxyadenosine. Blood. 1993 Feb 1;81(3):597-601.

14 Albertioni F, Lindemalm S, Reichelova V, Pettersson B, Eriksson S, Juliusson G, *et al*. Pharmacokinetics of cladribine in plasma and its 5'-monophosphate and 5'-triphosphate in leukemic cells of patients with chronic lymphocytic leukemia. Clin Cancer Res. 1998 Mar;4(3):653-8.

15 Leiby JM, Snider KM, Kraut EH, Metz EN, Malspeis L, Grever MR. Phase II trial of 9-beta-D-arabinofuranosyl-2-fluoroadenine 5'-monophosphate in non-Hodgkin's lymphoma: prospective comparison of response with deoxycytidine kinase activity. Cancer Res. 1987 May 15;47(10):2719-22.

16 Chottiner EG, Shewach DS, Datta NS, Ashcraft E, Gribbin D, Ginsburg D, *et al*. Cloning and expression of human deoxycytidine kinase cDNA. Proc Natl Acad Sci U S A. 1991 Feb 15;88(4):1531-5.

17 Antonsson BE, Avramis VI, Nyce J, Holcenberg JS. Effect of 5-azacytidine and congeners on DNA methylation and expression of deoxycytidine kinase in the human lymphoid cell lines CCRF/CEM/0 and CCRF/CEM/dCk-1. Cancer Res. 1987 Jul 15;47(14):3672-8.

18 Ueno H, Kiyosawa K, Kaniwa N. Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy? Br J Cancer. 2007 Jul 16;97(2):145-51.

19 Yue L, Saikawa Y, Ota K, Tanaka M, Nishimura R, Uehara T, *et al*. A functional single-nucleotide polymorphism in the human cytidine deaminase gene contributing to ara-C sensitivity. Pharmacogenetics.2003 Jan;13(1):29-38.

20 Fukunaga AK, Marsh S, Murry DJ, Hurley TD, McLeod HL. Identification and analysis of single-nucleotide polymorphisms in the gemcitabine pharmacologic pathway. Pharmacogenomics J. 2004;4(5):307-14.

21 Gilbert JA, Salavaggione OE, Ji Y, Pelleymounter LL, Eckloff BW, Wieben ED, *et al*. Gemcitabine pharmacogenomics: cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. Clin Cancer Res. 2006 Mar 15;12(6):1794-803.

22 Sugiyama E, Kaniwa N, Kim SR, Kikura-Hanajiri R, Hasegawa R, Maekawa K, *et al*. Pharmacokinetics of gemcitabine in Japanese cancer patients: the impact of a cytidine deaminase polymorphism J Clin Oncol. 2007 Jan 1 25(1):32-42.

23 Fitzgerald SM, Goyal RK, Osborne WR, Roy JD, Wilson JW, Ferrell RE. Identification of functional single nucleotide polymorphism haplotypes in the cytidine deaminase promoter. Hum Genet. 2006 Apr;119(3):276-83.

24 Yonemori K, Ueno H, Okusaka T, Yamamoto N, Ikeda M, Saijo N, *et al.* Severe drug toxicity associated with a single-nucleotide polymorphism of the cytidine deaminase gene in a Japanese cancer patient treated with gemcitabine plus cisplatin. Clin Cancer Res. 2005 Apr 1;11(7):2620-4.

25 Capizzi RL, White JC, Powell BL, Perrino F. Effect of dose on the pharmacokinetic and pharmacodynamic effects of cytarabine. Semin Hematol. 1991 Jul;28(3 Suppl 4):54-69.

26 Honma Y, Onozuka Y, Okabe-Kado J, Kasukabe T, Hozumi M. Hemin enhances the sensitivity of erythroleukemia cells to 1-beta-D-arabinofuranosylcytosine by both activation of deoxycytidine kinase and reduction of cytidine deaminase activity. Cancer Res. 1991 Sep 1;51(17):4535-8.

27 Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. Leukemia. 2001 Jun;15(6):875-90.

28 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, *et al*. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol. 1976 Aug;33(4):451-8.

29 Schmaier AH. Hematology for the medical student: Lippincott Williams & Wilkins 2003.

30 Jorge EC, Hagop MK. Acute lymphoblastic leukemia a comprehensive review with emphasis on biology and therapy. Cancer. 1995;76(12):2393-417.

31 Pui C-H. childhood leukemia. 2 ed: 2006.

31 Pui C-H, Campana D, Evans WE. Childhood acute lymphoblastic leukaemia - current status and future perspectives. The Lancet Oncology. 2001;2(10):597-607.

33 Lewis BS, Thomas WM, Richard DG, Mia JD, Gilliland DG, Nancy JT, *et al.* Intensified therapy for infants with acute lymphoblastic leukemia. 1997:2285-95.

34 Dordelmann M, Reiter A, Borkhardt A, Ludwig W-D, Gotz N, Viehmann S, *et al.* Prednisone Response Is the Strongest Predictor of Treatment Outcome in Infant Acute Lymphoblastic Leukemia. 1999:1209-17.

35 Sullivan MP, Pullen DJ, Crist WM, Brecher M, Ramirez I, Sabio H, *et al*. Clinical and biological heterogeneity of childhood B cell acute lymphocytic leukemia: implications for clinical trials. Leukemia. 1990 Jan;4(1):6-11.

36 Schrappe M, Reiter A, Zimmermann M, Harbott J, Ludwig WD, Henze G, *et al*. Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Munster. Leukemia. 2000 Dec;14(12):2205-22.

37 Gaynon PS, Trigg ME, Heerema NA, Sensel MG, Sather HN, Hammond GD, *et al*. Children's Cancer Group trials in childhood acute lymphoblastic leukemia: 1983-1995. Leukemia. 2000 Dec;14(12):2223-33.

38 Vilmer E, Suciu S, Ferster A, Bertrand Y, Cave H, Thyss A, *et al*. Long-term results of three randomized trials (58831, 58832,58881) in childhood acute lymphoblastic leukemia: a CLCG-EORTC report. Children Leukemia Cooperative Group. Leukemia. 2000 Dec;14(12):2257-66.

39 Maloney KW, Shuster JJ, Murphy S, Pullen J, Camitta BA. Long-term results of treatment studies for childhood acute lymphoblastic leukemia: Pediatric Oncology Group studies from 1986-1994. Leukemia. 2000 Dec;14(12):2276-85.

40 Pui CH, Boyett JM, Rivera GK, Hancock ML, Sandlund JT, Ribeiro RC, *et al*. Long-term results of Total Therapy studies 11,12 and 13A for childhood acute lymphoblastic leukemia at St Jude Children's Research Hospital. Leukemia. 2000 Dec;14(12):2286-94.

41 Tsuchida M, Ikuta K, Hanada R, Saito T, Isoyama K, Sugita K, *et al.* Long-term follow-up of childhood acute lymphoblastic leukemia in Tokyo Children's Cancer Study Group 1981-1995. Leukemia. 2000 Dec;14(12):2295-306.

42 Eden OB, Harrison G, Richards S, Lilleyman JS, Bailey CC, Chessells JM, *et al.* Long-term follow-up of the United Kingdom Medical Research Council protocols for childhood acute lymphoblastic leukaemia, 1980-1997. Medical Research Council Childhood Leukaemia Working Party. Leukemia. 2000 Dec;14(12):2307-20.

43 Pui C-H, Evans WE. Treatment of Acute Lymphoblastic Leukemia. N Engl J Med. 2006 January 12, 2006;354(2):166-78.

44 Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, *et al*. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446(7137):758-64.

45 Grant S. Ara-C: cellular and molecular pharmacology. Adv Cancer Res. 1998;72:197-233.

46 Kanno S-i, Hiura T, Ohtake T, Koiwai K, Suzuki H, Ujibe M, *et al.* Characterization of resistance to cytosine arabinoside (Ara-C) in NALM-6 human B leukemia cells. Clinica Chimica Acta. 2007;377(1-2):144-9.

47 Ross DD, Thompson BW, Joneckis CC, Akman SA, Schiffer CA. Metabolism of ara-C by blast cells from patients with ANLL.Blood.1986Jul;68(1):76-82.

48 Mahlknecht U, Dransfeld CL, Bulut N, Kramer M, Thiede C, Ehninger G, *et al.* SNP analyses in cytarabine metabolizing enzymes in AML patients and their impact on treatment response and patient survival: identification of CDA SNP C-451T as an independent prognostic parameter for survival. Leukemia. 2009.

49 Wiley JS, Jones SP, Sawyer WH, Paterson AR. Cytosine arabinoside influx and nucleoside transport sites in acute leukemia. J Clin Invest. 1982 Feb;69(2):479-89.

50 White JC, Rathmell JP, Capizzi RL. Membrane transport influences the rate of accumulation of cytosine arabinoside in human leukemia cells. J Clin Invest. 1987 Feb;79(2):380-7.

51 Mata JF, Scrideli CA, Queiroz RP, Mori BO, Emerenciano M, Pombo-de-Oliveira MS, *et al.* Cytosine arabinoside-metabolizing enzyme genes are underexpressed in children with MLL gene-rearranged acute lymphoblastic leukemia. Braz J Med Biol Res. 2006 Nov;39(11):1417-23.

52 Stam RW, den Boer ML, Meijerink JP, Ebus ME, Peters GJ, Noordhuis P, *et al.* Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood. 2003 Feb 15;101(4):1270-6.

53 Kufe D, Spriggs D, Egan EM, Munroe D. Relationships among Ara-CTP pools, formation of (Ara-C)DNA, and cytotoxicity of human leukemic cells. Blood. 1984 Jul;64(1):54-8.

54 Neff T, Blau CA. Forced expression of cytidine deaminase confers resistance to cytosine arabinoside and gemcitabine. Exp Hematol. 1996 Sep;24(11):1340-6.

55 Jan KS, Seidelmann M, Hans-Christoph K, Siegfried S, Jochen S. Assessment of resistance induction to cytosine arabinoside following transfer and overexpression of the deoxycytidylate deaminase gene in vitro. Leukemia research. 1998;22(7):619-24.

56 Isabelle Hubeek2 G-JLK, Gert J. Ossenkoppele4 and Godefridus J. Peters5. Cytosine Arabinoside Deoxynucleoside Analogs In Cancer Therapy. 2007:119-52.

57 Kufe D, Spriggs D, Egan EM, Munroe D. Relationships among Ara-CTP pools, formation of (Ara-C)DNA, and cytotoxicity of human leukemic cells. 1984:54-8.

58 Momparler RL, Onetto-Pothier N, Bouffard DY, Momparler LF. Cellular pharmacology of 1-beta-D-arabinofuranosylcytosine in human myeloid, B-lymphoid and T-lymphoid leukemic cells. Cancer Chemother Pharmacol. 1990;27(2):141-6.

59 Plunkett W, Hug V, Keating MJ, Chubb S. Quantitation of 1-{beta}-D-Arabinofuranosylcytosine 5'-Triphosphate in the Leukemic Cells from Bone Marrow and Peripheral Blood of Patients Receiving 1-{beta}-D-Arabinofuranosylcytosine Therapy. 1980:588-91.

60 Colly LP, Richel DJ, Arentsen-Honders W, Starrenburg IW, Edelbroek PM, Willemze R. A simplified assay for measurement of cytosine arabinoside incorporation into DNA in Ara-C-sensitive and -resistant leukemic cells. Cancer Chemother Pharmacol. 1990;27(2):151-6.

61 Rustum YM, Danhauser L, Luccioni C, Au JL. Determinants of response to antimetabolites and their modulation by normal purine and pyrimidine metabolites. Cancer Treat Rep. 1981;65 Suppl 3:73-82.

62 Preisler HD, Rustum Y, Priore RL. Relationship between leukemic cell retention of cytosine arabinoside triphosphate and the duration of remission in patients with acute non-lymphocytic leukemia. Eur J Cancer Clin Oncol. 1985 Jan;21(1):23-30.

63 Bolwell BJ, Cassileth PA, Gale RP. High dose cytarabine: a review. Leukemia. 1988 May;2(5):253-60.

64 Peters GJ, Schornagel JH, Milano GA. Clinical pharmacokinetics of anti-metabolites. Cancer Surv. 1993;17:123-56.

65 Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues and nucleobases in cancer treatment. The Lancet Oncology. 2002;3(7):415-24.

66 Fleming RA, Capizzi RL, Rosner GL, Oliver LK, Smith SJ, Schiffer CA, *et al*. Clinical pharmacology of cytarabine in patients with acute myeloid leukemia: a cancer and leukemia group B study. Cancer Chemother Pharmacol. 1995;36(5):425-30.

67 www.fda.gov/MedWatch. Ara-C.

68 Lamba JK, Crews K, Pounds S, Schuetz EG, Gresham J, Gandhi V, *et al.* Pharmacogenetics of deoxycytidine kinase: identification and characterization of novel genetic variants. J Pharmacol Exp Ther. 2007 Dec;323(3):935-45.

69 Carson DA, Wasson DB, Kaye J, Ullman B, Martin DW, Jr., Robins RK, *et al.* Deoxycytidine kinase-mediated toxicity of deoxyadenosine analogs toward malignant human lymphoblasts in vitro and toward murine L1210 leukemia in vivo. Proc Natl Acad Sci U S A. 1980 Nov;77(11):6865-9.

70 Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the Cellular Pharmacokinetics and Toxicity of 2',2'-Difluorodeoxycytidine and 1-{beta}-D-Arabinofuranosylcytosine. 1988:4024-31.

71 Coleman CN, Stoller RG, Drake JC, Chabner BA. Deoxycytidine kinase: properties of the enzyme from human leukemic granulocytes.1975:791-803.

72 Kawasaki H, Shindou K, Higashigawa M, Cao D-C, Hori H, Ido M, *et al.* Deoxycytidine kinase mRNA Levels in leukemia cells with competitive polymerase chain reaction assay. Leukemia Research. 1996;20(8):677-82.

73 Stegmann AP, Honders MW, Kester MG, Landegent JE, Willemze R. Role of deoxycytidine kinase in an in vitro model for AraC- and DAC-resistance: substrate-enzyme interactions with deoxycytidine, 1-beta-D-arabinofuranosylcytosine and 5-aza-2'-deoxycytidine. Leukemia. 1993 Jul;7(7):1005-11.

74 Charles D, Krystyna F-M, Danko M, Evelyne Callet B, Isabelle T, Varsha G, *et al.* Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. 1999:78-85.

75 Owens JK, Shewach DS, Ullman B, Mitchell BS. Resistance to 1-{beta}-D-Arabinofuranosylcytosine in Human T-Lymphoblasts Mediated by Mutations within the Deoxycytidine Kinase Gene. 1992:2389-93.

76 Veuger MJT, Honders MW, Landegent JE, Willemze R, Barge RMY. High incidence of alternatively spliced forms of deoxycytidine kinase in patients with resistant acute myeloid leukemia. 2000:1517-24.

77 Gregoire V, Rosier J-F, De Bast M, Bruniaux M, De Coster B, Octave-Prignot M, *et al*. Role of deoxycytidine kinase (dCK) activity in gemcitabine's radioenhancement in mice and human cell lines in vitro. Radiotherapy and Oncology. 2002;63(3):329-38.

78 Bergman AM, Pinedo HM, Jongsma APM, Brouwer M, Ruiz van Haperen VWT, Veerman G, *et al*. Decreased resistance to gemcitabine (2',2'-difluorodeoxycitidine) of cytosine arabinoside-resistant myeloblastic murine and rat leukemia cell lines: role of altered activity and substrate specificity of deoxycytidine kinase. Biochemical Pharmacology. 1999;57(4):397-406.

79 van Haperen VWTR, Veerman G, Eriksson S, Boven E, Stegmann APA, Hermsen M, *et al*. Development and Molecular Characterization of a 2',2'-Difluorodeoxycytidine-resistant Variant of the Human Ovarian Carcinoma Cell Line A2780. 1994:4138-43.

80 Ruiz van Haperen VW, Veerman G, Eriksson S, Stegmann AP, Peters GJ. Induction of resistance to 2',2'-difluorodeoxycytidine in the human ovarian cancer cell line A2780. Semin Oncol. 1995 Aug;22(4 Suppl 11):35-41.

81 Stegmann AP, Honders MW, Hagemeijer A, Hoebee B, Willemze R, Landegent JE. In vitro-induced resistance to the deoxycytidine analogues cytarabine (AraC) and 5-aza-2'-deoxycytidine (DAC) in a rat model for acute myeloid leukemia is mediated by mutations in the deoxycytidine kinase (dck) gene. Ann Hematol. 1995 Jul;71(1):41-7.

82 Ge Y, Jensen TL, Stout ML, Flatley RM, Grohar PJ, Ravindranath Y, *et al*. The role of cytidine deaminase and GATA1 mutations in the increased cytosine arabinoside sensitivity of Down syndrome myeloblasts and leukemia cell lines. Cancer Res. 2004 Jan 15;64(2):728-35.

83 Momparler RL, Laliberte J, Eliopoulos N, Beausejour C, Cournoyer D. Transfection of murine fibroblast cells with human cytidine deaminase cDNA confers resistance to cytosine arabinoside. Anticancer Drugs. 1996 May;7(3):266-74.

84 Schroder JK, Kirch C, Flasshove M, Kalweit H, Seidelmann M, Hilger R, *et al*. Constitutive overexpression of the cytidine deaminase gene confers resistance to cytosine arabinoside in vitro. Leukemia. 1996 Dec;10(12):1919-24.

85 Najafi M, Firoozrai M, Gohari HL, Zavarehie A, Basiri G. Direct haplotyping of bi-allelic SNPs using ARMS and RFLP analysis techniques. Biomolecular Engineering. 2007;24(6):609-12.

86 Wang DG, Fan J-B, Siao C-J, Berno A, Young P, Sapolsky R, *et al*. Large-Scale Identification, Mapping, and Genotyping of Single-Nucleotide Polymorphisms in the Human Genome. 1998:1077-82.

87 Shields PG, Harris CC. Molecular Epidemiology and the Genetics of Environmental Cancer. 1991:681-7

88 Shields PG. Inherited factors and environmental exposures in cancer risk. J Occup Med. 1993 Jan;35(1):34-41.

89 Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). 1989:2503-16.

90 Old JM. Detection of Mutations by the Amplification Refractory Mutation System (ARMS). *Protocols in Human Molecular Genetics* 1992:77-84.

91 van der Wilt CL, Kroep JR, Loves WJP, Rots MG, Van Groeningen CJ, Kaspers GJ, *et al*. Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver. European Journal of Cancer. 2003;39(5):691-7.

92 Evans WE, Relling MV. Pharmacogenomics: Translating Functional Genomics into Rational Therapeutics. 1999:487-91.

93 Relling MV, Dervieux T. Pharmacogenetics and cancer therapy. Nat Rev Cancer. 2001;1(2):99-108.

94 Meyers MB, Kreis W. Comparison of Enzymatic Activities of Two Deoxycytidine Kinases Purified from Cells Sensitive (P815) or Resistant (P815/ara-C) to 1-{beta}-D-Arabinofuranosylcytosine. 1978:1105-12.

95 Bhalla K, Nayak R, Grant S. Isolation and Characterization of a Deoxycytidine Kinase-deficient Human Promyelocytic Leukemic Cell Line Highly Resistant to 1-{beta}-D- Arabinofuranosylcytosine. 1984:5029-37.

96 Richel DJ, Colly LP, Arkesteijn GJA, Arentsen-Honders MW, Kerster MGD, ter Riet PM, *et al.* Substrate-specific Deoxycytidine Kinase Deficiency in 1-{beta}-D-Arabinofuranosylcytosine-resistant Leukemic Cells.1990:6515-9.

97 Kees UR, Ford J, Dawson VM, Piall E, Wynne Aherne G. Development of Resistance to 1-{beta}-D-Arabinofuranosylcytosine after High-Dose Treatment in Childhood Lymphoblastic Leukemia: Analysis of Resistance Mechanism in Established Cell Lines. 1989:3015-9.

98 Yusa K, Oh-hara T, Tsukahara S, Tsuruo T. Human immunodeficiency virus type 1 induces 1-beta-D-arabinofuranosylcytosine resistance in human H9 cell line. J Biol Chem. 1992 Aug 25;267(24):16848-50.

99 Riva C, Barra Y, Carcassonne Y, Cano JP, Rustum Y. Effect of tetrahydrouridine on metabolism and transport of 1-beta-D-arabinofuranosylcytosine in human cells. Chemotherapy. 1992;38(5):358-66.

100 Kreis W, Hession C, Soricelli A, Scully K. Combinations of tetrahydrouridine and cytosine arabinoside in mouse tumors. Cancer Treat Rep. 1977 Oct;61(7):1355-64.

101 Sebastiani V, Ricci F, Rubio-Viqueira B, Kulesza P, Yeo CJ, Hidalgo M, *et al.* Immunohistochemical and genetic evaluation of deoxycytidine kinase in pancreatic cancer: relationship to molecular mechanisms of gemcitabine resistance and survival. Clin Cancer Res. 2006 Apr 15;12(8):2492-7.

102 Kaspers GJ, Zwaan CM, Pieters R, Veerman AJ. Cellular drug resistance in childhood acute myeloid leukemia. A mini-review with emphasis on cell culture assays. Adv Exp Med Biol. 1999;457:415-21.

103 Zwaan CM, Kaspers GJL, Pieters R, Hahlen K, Janka-Schaub GE, van Zantwijk CH, *et al.* Different drug sensitivity profiles of acute myeloid and lymphoblastic leukemia and normal peripheral blood mononuclear cells in children with and without Down syndrome. 2002:245-51.

104 Dumontet C, Fabianowska-Majewska K, Mantincic D, Callet Bauchu E, Tigaud I, Gandhi V, *et al.* Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol. 1999 Jul;106(1):78-85.

105 Kobayashi T, Kakihara T, Uchiyama M, Fukuda T, Kishi K, Shibata A. Low expression of the deoxycytidine kinase (dCK) gene in a 1-beta-D-arabinofuranosylcytosine-resistant human leukemic cell line KY-Ra. Leuk Lymphoma. 1994 Nov;15(5-6):503-5.

106 Kirch HC, Schroder J, Hoppe H, Esche H, Seeber S, Schutte J. Recombinant gene products of two natural variants of the human cytidine deaminase gene confer different deamination rates of cytarabine in vitro. Exp Hematol. 1998 May;26(5):421-5.

107 Bhatla D, Gerbing RB, Alonzo TA, Conner H, Ross JA, Meshinchi S, *et al.* Cytidine deaminase genotype and toxicity of cytosine arabinoside therapy in children with acute myeloid leukemia. Br J Haematol. 2008 Nov 22.

108 Bengala C, Guarneri V, Giovannetti E, Lencioni M, Fontana E, Mey V, *et al.* Prolonged fixed dose rate infusion of gemcitabine with autologous haemopoietic support in advanced pancreatic adenocarcinoma. Br J Cancer. 2005 Jul 11;93(1):35-40.

109 Jordheim LP, Dumontet C. Review of recent studies on resistance to cytotoxic deoxynucleoside analogues. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2007;1776(2):138-59.

APPENDIX

Solution for Electrophoresis and ethidium bromide staining

1. 10X TBE (stock solution)

Makes 1 L. Store at room temperature indefinitely.

- 1 g of NaOH
- 108 g of Tris base (m.w. 121.10)
- 55 g of boric acid (m.w. 61.83)
- 9.5 g of ethylene diamine tetraacetic acid (EDTA, disodium salt, m.w. 372.24)

Add all dry ingredients to 700 mL of deionized or distilled water in a 2 L flask. Stirile to dissolve, preferably using a magnetic stirrer until the dry was dissolved and added distilled water to bring the total solution to 1L.

Working solution (1X TBE) was prepared by adding 100 ml of 10X TBE with 900 ml of DW.

2. 50 mg/ml Ethidium bromide 100 ml

1. Ethidium bromide	0.5	g
2. Distilled water	100	ml

Dissolved ethidium bromide with distill water 100 ml (5mg/ml) and sterile on magnetic sterer until the dry was dissolved. Store in the dark bottle and keep at room temperature.

BIOGRAPHY

NAME	Miss Chumphorn Banklaui
DATE OF BIRTH	26 January 1979
PLACE OF BIRTH	Kanchanaburi, Thailand
INSTITUTIONS ATTENDED	Thammasat University, 1998–2002:
	Bachelor of Science
	(Medical Technology)
	Mahidol University, 2007–2009:
	Master of Science
	(Clinical Pathology)
RESEARCH GRANT	Supported in part by the Thesis Grant,
	Faculty of Graduate Studies,
	Mahidol University, Bangkok Thailand
HOME ADDRESS	19>22 Nongkhoaw District,
	Amphur Thamuang, kanchanaburi Province,
	Thailand 71110
	E-mail: c_noi1@hotmail.com