MOLECULAR CHARACTERIZATION OF *AML1* GENE IN ACUTE MYELOID LEUKEMIA

AMPORN LEECHARENDKEAT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE (IMMUNOLOGY)

FACULTY OF GRADUATE STUDIES

MAHIDOL UNIVERSITY

2006

ISBN 974-04-7040-8

Thesis
Entitled

MOLECULAR CHARACTERIZATION OF *AML1* GENE IN ACUTE MYELOID LEUKEMIA

Miss Amporn Leecharendkeat
Candidate

Assoc.Prof. Chirayu Auewarakul,
M.D., Ph.D., Dip. Amer. Brd. Int. Med.,
Dip. Amer. Brd. Hematol
Major Advisor

Prof. Napatawn Banchuin,
M.D., Ph.D.
Co-Advisor

Prof. Pa-thai Yenchitsomanus,
Ph.D.
Co-Advisor

Dr. Wanna Thongnoppakhun
Ph.D.
Co-Advisor

Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies

Prof. Kovit Pattanapanyasat,
Ph.D.
Chairman
Master of Science Programme in
Immunology
Faculty of Medicine Siriraj Hospital

Thesis
Entitled

MOLECULAR CHARACTERIZATION OF *AML1* GENE IN ACUTE MYELOID LEUKEMIA

was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Master of Science (Immunology)
on
3 April, 2006

Amporn Leecharendkeat
Miss Amporn Leecharendkeat
Candidate

Napatawn Banchuin
Prof. Napatawn Banchuin,
MD, Ph.D.
Member

Chirayu Auewarakul
Assoc.Prof. Chirayu Auewarakul,
M.D, Ph.D.
Chair

Chanin Limwongse
Lect. Chanin Limwongse, MD,
Dip Amer Brd Int Med,
Dip Amer Brd Med Genet
Member

Pa-thai Yenchitsomanus
Prof. Pa-thai Yenchitsomanus,
Ph.D.
Member

C. Tocharoentanaphol
Assist.Prof. Chintana Tocharoentanaphol,
Ph.D.
Member

Wanna Thongnoppakhun
Dr. Wanna Thongnoppakhun
Ph.D.
Member

Jisnuson Svasti
Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

P. Sakolsatayadorn
Clin.Prof. Piyasakol Sakolsatayadorn,
M.D., FRCST
Dean
Faculty of Medicine, Siriraj Hospital
Mahidol University

ACKNOWLEDGEMENTS

During the years of my master degree study, I gained a lot of experiences and knowledge in scientific and research activities through the kindness of so many people who supported and assisted me. I would therefore firstly express my deepest and sincere gratitude to my major-advisor Assoc. Prof. Dr. Chirayu Auewarakul, for accepting me into the master program of the Department of Immunology and also for taking a best care for me. I am extremely grateful to my co-advisors, Assoc. Prof. Dr. Pa-thai Yenchitsommanus, Prof. Dr. Napatawn Banchuin, and Dr. Wanna Thongnoppakhun, for their guidance, invaluable advice, instruction, supervision and other interactions that kept this work going throughout this study. Equally, my great thanks are given to the external committees, Dr. Chanin Limwongse and Dr. Chintana Tocharoentanaphol, for their very invaluable instructions on my thesis examination and correction.

My great thanks are given to the staffs at the Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, for the excellent care of the patients in this study and their help with leukemia sample collection and processing. Particularly, I am thankful to Ms. Orathai Promsuwicha, Ms. Kunjirat Promroeng and Ms. Darat Lauhakirte for their hard work with flow cytometric analysis. Also, I wish to thank to Mr. Narongrit Sritana for his hard work in data collection and set up of *FLT3* mutation analysis.

My sincere appreciation is expressed to and all staffs in the Molecular Genetic Unit (MGU), Faculty of Medicine Siriraj Hospital for their kindness, continuous guidance, and encouragement throughout my study. I also appreciated the staffs and graduate students in the Department of Immunology for their friendship and kind support. Very special thank is devoted to my family for their love and understanding.

Finally, I wish to thank Faculty of Medicine Siriraj Hospital Graduate Studies Scholarship for their financial support.

Amporn Leecharendkea

MOLECULAR CHARACTERIZATION OF *AML1* GENE IN ACUTE MYELOID LEUKEMIA

AMPORN LEECHARENDKEAT 4636407 SIIM/M

M.Sc. (IMMUNOLOGY)

THESIS ADVISORS: CHIRAYU AUEWARAKUL, M.D., Ph.D., PA-THAI YENCHITSOMANUS, Ph.D., WANNA THONGNOPPAKHUN, Ph.D., NAPATAWN BANCHUIN, M.D., Ph.D.

ABSTRACT

AML1 gene belongs to a class of transcription factor genes frequently deregulated in leukemia. The objectives of this study are: 1) to develop the molecular assays for the detection of genetic abnormalities involving *AML1* gene, particularly, *AML1-ETO* gene rearrangement and *AML1* point mutation, 2) to determine the incidence of *AML1-ETO* gene rearrangement and type of *AML1* point mutation in Thai adult *de novo* acute myeloid leukemia (AML) population, 3) to explore if *AML1-ETO* gene rearrangement and *AML1* point mutation have any unique association with patients' clinical features and immunophenotypes, and 4) to determine if *FLT3* tyrosine kinase receptor mutation coexists in patients with *AML1* gene abnormalities.

Semi nested reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect *AML1-ETO* fusion gene generated by t(8;21). *AML1* mutations of exons 3, 4, and 5 were screened by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis followed by sequencing analysis. *FLT3* mutations were analyzed by standard PCR analysis to detect *FLT3* internal tandem duplication (ITD) mutation. *FLT3*-tyrosine kinase domain (TKD) mutation in codon 835 was screened by restriction fragment length polymorphism (RFLP) analysis using *Eco*RV enzyme. Immunophenotyping and karyotyping of leukemic cells were carried out according to the standard methods.

AML1-ETO fusion gene was detected in 16 of 108 studied cases (15%) and predominantly found in AML-M2 subtype (38%). AML-M2 patients with *AML1-ETO* were significantly younger than patients without the fusion gene (average 36 vs 43, respectively) ($p<0.05$). Eighty-six percent of patients with *AML1-ETO* expressed CD56 antigen as compared to 47% of those without ($p<0.05$). CD117 and CD34 antigens were also highly expressed in patients with *AML1-ETO*. AML-M2 patients with t(8;21) and *AML1-ETO* fusion gene had a high rate of sex chromosome loss (41%) as compared to other subtypes.

Sixteen of 414 patients (4%) had *AML1* mutation. The majority were young adults predominantly categorized as M4, M5, or M6 (50%). Interestingly, the highest incidence of *AML1* mutation in the Thai population was found in non-M0 subtype, i.e. AML-M6 (25%) followed by M5 (6.3%) and M4 (6%), respectively. Eight novel and four known mutations were found. Five novel mutations were found in exon 4 including c.341C>T, c.416_417ins9, c.422_423ins9, c.292delC and c.359C>A with one novel mutation, c.231_232dupGC, in exon 3. Additionally, a novel duplication of 5 nucleotides in intron 3 (dup5 in IVS3-5) and a deletion of 17 nucleotides in intron 5 (del17 in IVS5+41) were also found. Four known mutations were c.238C>T, c.210delC, c.343_364dup22, and c.524G>A. Half of the mutated *AML1* cases had coexisting chromosomal abnormalities including t(16;21), t(8;21), 21q+, +8, -7, del(5q), and del(9q). *FLT3* mutation was found frequently in patients harboring *AML1* point mutation (25%) but in only 6.25% of patients with *AML1-ETO*.

In conclusion, deregulation mechanisms of *AML1* gene in Thai adult AML patients were identified, i.e. translocation and point mutation. All cases with t(8;21) had detectable *AML1-ETO* by RT-PCR assay. CD56 antigen was found to be associated with the presence of *AML1-ETO* gene. Eight novel mutations of *AML1* gene were discovered. As no other reports described *AML1* mutation in M6 patients, this study represents the first series to report two cases of AML-M6 with *AML1* mutations, both with a novel mutation. The prognostic significance of these novel mutations should be further determined in a larger population. The current molecular methods that were developed for the detection of *AML1-ETO* fusion gene and *AML1* mutation in this study should be of value for future molecular classification and genetic risk-stratification of adult AML patients in Thailand.

KEY WORDS: *AML1* MUTATION/ *AML1-ETO* FUSION GENE/ ACUTE MYELOID LEUKEMIA
130 P. ISBN 974-04-7040-8

การศึกษาลักษณะระดับโมเลกุลของยีน *AML1* ในโรคมะเร็งเม็ดเลือดขาวสายมัยอีลอยด์ชนิดเฉียบพลัน

(MOLECULAR CHARACTERIZATION OF *AML1* GENE IN ACUTE MYELOID LEUKEMIA)

อำพร ลีเจริญเกียรติ 4636407 SIIM/M

วท.ม. (วิทยาภูมิคุ้มกัน)

คณะกรรมการควบคุมวิทยานิพนธ์ : จิรายุ เอื้อวรากุล, พ.บ., Ph.D., เพทาย เย็นจิตโสมนัส, Ph.D., วรรณา ทองนพคุณ, Ph.D., นภาธร บานชื่น, พ.บ., Ph.D.

บทคัดย่อ

ยีน *AML1* เป็นหนึ่งในกลุ่มยีน transcription factor ที่พบบ่อยในผู้ป่วยโรคมะเร็งเม็ดเลือด วัตถุประสงค์ของการศึกษานี้ได้แก่ 1) พัฒนาเทคนิคด้านอณูชีววิทยาเพื่อใช้ในการศึกษาความผิดปกติที่เกี่ยวข้องกับยีน *AML1* 2) เพื่อหาอุบัติการณ์ของยีนลูกผสม *AML1-ETO* และ การกลายพันธุ์ของยีน *AML1* 3) เพื่อหาความสัมพันธ์ของยีนลูกผสม *AML1-ETO* และ การกลายพันธุ์ของยีน *AML1* ต่อลักษณะทางคลินิกและ ผลอิมมูโนฟีโนทัยป์ของผู้ป่วย และ 4) เพื่อศึกษาความสัมพันธ์ระหว่างความผิดปกติของยีน *FLT3* ซึ่งเป็นอยู่ในกลุ่ม tyrosine kinase receptor กับความผิดปกติของยีน *AML1*

เทคนิค semi nested reverse transcriptase-polymerase chain reaction (RT-PCR) ได้ถูกพัฒนาเพื่อใช้ตรวจยีนลูกผสม *AML1-ETO* ซึ่งเกิดจากการแลกเปลี่ยนชิ้นส่วนระหว่างโครโมโซมที่ 8 กับ 21 ส่วนการตรวจการกลายพันธุ์ของยีน *AML1* ใน exon 3, 4, และ 5 ใช้เทคนิค polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) การตรวจความผิดปกติของยีน *FLT3* ที่เป็น internal tandem duplication (*FLT3* ITD) อาศัยวิธีพีซี-อาร์ และการตรวจการกลายพันธ์ของยีน tyrosine kinase domain (*FLT3*-TKD) ที่ตำแหน่ง codon 835 ใช้วิธี restriction fragment length polymorphism (RFLP) โดยย่อยด้วยเอนไซม์ตัดจำเพาะ *EcoR*V การตรวจลักษณะของเซลล์มะเร็งในแง่ชนิดของแอนติเจนบนผิวเซลล์ (Immunophenotype) และการตรวจโครโมโซมทำตามวิธีมาตรฐาน

ผลการศึกษาพบอุบัติการณ์ของยีนลูกผสม *AML1-ETO* 16 ราย จากผู้ป่วยทั้งหมด 108 ราย (ร้อยละ 15) ซึ่งพบมากในกลุ่มย่อย AML-M2 (ร้อยละ 38%) ผู้ป่วยกลุ่มย่อย AML-M2 ที่ตรวจพบยีนลูกผสมนี้ส่วนใหญ่จะมีอายุน้อยกว่ากลุ่มที่ไม่พบอย่างมีนัยสำคัญ (อายุเฉลี่ย 36 ต่อ 43 ปี ตามลำดับ) ($p<0.05$) ร้อยละ 86 ของผู้ป่วยกลุ่มย่อย AML-M2 ที่ตรวจพบยีนลูกผสม *AML1-ETO* มีการแสดงออกของแอนติเจนชนิด CD56 บนผิวเซลล์ซึ่งเป็นสัดส่วนที่สูงกว่ากรณีของผู้ป่วยที่ไม่พบยีนลูกผสมที่มีการแสดงออกเพียงร้อยละ 47 ($p<0.05$) นอกจากนี้ยังพบว่ามีการแสดงออกของ CD117 และ CD56 สูงกว่าด้วย การขาดหายไปของโครโมโซมเพศในผู้ป่วย AML-M2 ที่พบการแลกเปลี่ยนชิ้นส่วนระหว่างโครโมโซมที่ 8 กับ 21 ร่วมกับ *AML1-ETO* พบ ในอัตราที่สูงกว่ากลุ่มอื่น (ร้อยละ41)

อุบัติการณ์การเกิดการกลายพันธุ์ของยีน *AML1* เท่ากับร้อยละ 4 โดยพบในผู้ป่วย 16 ราย จากทั้งหมด 414 ราย ผู้ป่วยที่พบความผิดปกติส่วนใหญ่อายุน้อยและพบมากในชนิดย่อย M4, M5, หรือ M6 (ร้อยละ 50) เป็นที่น่าสนใจว่าการกลายพันธุ์ของยีน *AML1* พบมากในผู้ป่วยที่ไม่ใช่ AML-M0 คือพบมากที่สุดใน M6 (ร้อยละ 25) ตามด้วย M5 (ร้อยละ 6.3) และ M4 (ร้อยละ 6) ตามลำดับ ในการศึกษานี้พบความผิดปกติตำแหน่งใหม่ที่ยังไม่เคยมีรายงานมาก่อน 8 ตำแหน่ง และที่เคยรายงานมาแล้ว 4 ตำแหน่ง ความผิดปกติใหม่ 5 ตำแหน่งที่พบใน exon 4 ประกอบด้วย c.341C>T, c.416_417ins9, c.422_423ins9, c.292delC และ c.359C>A และพบความผิดปกติใหม่ใน exon 3 ตำแหน่งเดียวคือ c.231_232dupGC นอกจากนี้ยังพบการเพิ่มนิวคลีโอไทด์ 5 ตัวในส่วน intron 3 และพบการขาดหายของนิวคลีโอไทด์ 17 ตัว ที่ intron 5 ส่วนความผิดปกติที่เคยมีรายงานมาแล้วได้แก่ c.238C>T, c.210delC, c.343_364dup22 และ c.524G>A พบความผิดปกติระดับโครโมโซมชนิดอื่นเกิดร่วมด้วยในครึ่งหนึ่งของผู้ป่วยทั้งหมดที่พบการกลายพันธุ์ของยีน *AML1* ได้แก่ การแลกเปลี่ยนชิ้นส่วนระหว่างโครโมโซม 16 กับ 21 และ 8 กับ 21, +8, -7 , 21q+ และการขาดหายไปบางส่วนของโครโมโซม 5 หรือ 9 ความผิดปกติของยีน *FLT3* จะพบบ่อยในผู้ป่วยที่พบการกลายพันธุ์ของยีน *AML1* (ร้อยละ 25) แต่ในผู้ป่วยที่พบยีนลูกผสม *AML1-ETO* พบความผิดปกติของยีน *FLT3* เพียงร้อยละ 6.25

โดยสรุปแล้ว กลไกที่ทำให้ยีน *AML1* เกิดความผิดปกติซึ่งได้แก่ การแลกเปลี่ยนชิ้นส่วนโครโมโซมและการกลายพันธ์สามารถพบได้ในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันในประเทศไทย ผู้ป่วยทุกรายที่พบการแลกเปลี่ยนชิ้นส่วนระหว่างโครโมโซม 8 กับ 21จะสามารถตรวจพบยีนลูกผสม *AML1-ETO* โดยวิธีอาร์ที-พีซีอาร์ การแสดงออกของแอนติเจน CD56 มีความสัมพันธ์กับการพบยีนลูกผสม *AML1-ETO* ในรายงานนี้พบการเกิดการกลายพันธ์ตำแหน่งใหม่ของยีน *AML1* 8 ตำแหน่ง และเป็นรายงานแรกที่พบการกลายพันธุ์ของยีน *AML1* ในผู้ป่วย M6 จำนวน 2 ราย ซึ่งเป็นความผิดปกติใหม่ทั้งคู่ ผลของการการกลายพันธุ์ใหม่ที่พบต่อการพยากรณ์ของโรคควรจะได้รับการศึกษาในประชากรกลุ่มใหญ่ต่อไปในอนาคต ขณะนี้ได้มีการพัฒนาวิธีการตรวจยีนลูกผสม *AML1-ETO* และ การกลายพันธุ์ของยีน *AML1* ขึ้นแล้วซึ่งจะเป็นประโยชน์อย่างยิ่งต่อการตรวจวินิจฉัยแยกชนิดย่อยของโรค และใช้เป็นตัวบ่งชี้การพยากรณ์โรคในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันในประเทศไทย

130 หน้า ISBN 974-04-7040-8

CONTENTS

CONTENTS (cont.)

Page

CONTENTS (cont.)

Page

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLE (cont.)

Table **Page**

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

Abbreviations		Term
ALL	=	Acute lymphoid leukemia
AML	=	Acute myeloid leukemia
BM	=	Bone marrow
bp	=	base pair
°C	=	degree Celsius
CBF	=	Core binding factor
dATP	=	deoxyadenosine-5’-triphosphate
dCTP	=	deoxycytosine-5’-triphosphate
dGTP	=	deoxyguanosine-5’-triphosphate
dTTP	=	deoxythymidine-5’-triphosphate
DNA	=	deoxyribonucleic acid
EDTA	=	ethylenediamine tetraacetic acid
ETO	=	Eight twenty one
FAB	=	French American British
kb	=	kilobase
M	=	molar
mg	=	milligram
ml	=	millilitre
mM	=	millimolar
MNC	=	mononuclear cell
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
ng	=	nanogram
nm	=	nanomolar
nt	=	nucleotide

LIST OF ABBREVIATIONS (cont.)

Abbreviations		Term
OD	=	optical density
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
pmol	=	picomolar
RNA	=	ribonucleic acid
rpm	=	revolutions per minute
RT-PCR	=	reverse transcriptase reaction
RUNX1	=	Runt-related protein 1
SSCP	=	single strand conformational polymorphism
Taq	=	*Thermus aquaticus*
TE	=	Tris-EDTA buffer
TBE	=	Tris-borate EDTA buffer
Tm	=	melting temperature
U	=	unit
WHO	=	World Health Organization

CHAPTER I
INTRODUCTION

Acute myeloid leukemia (AML) is the most common type of leukemia in Thai adults and one of the most common hematologic malignancies in the world (1). The hallmark of AML is the accumulation of immature "blast" cells in the bone marrow that leads to failure of bone marrow to produce normal blood elements (2, 3). AML patients thus suffer from anemia, bleeding or infections as a result of bone marrow failure (3).

The classification of acute leukemia has long been based on morphological criteria according to the French-American-British (FAB) Classification Sytem (1976) (4). However, the World Health Organization (WHO) has recently acknowledged the importance of cytogenetic and molecular genetic abnormalities of leukemic cells in the initial diagnosis and risk stratification of AML patients (5, 6). This is due to the fact that a high percentage of AML patients regardless of ethnic origin have been found to carry non-random chromosomal translocations, which subsequently led to the discovery of novel and known genes whose functions were disrupted as a result of chromosome breakage (4, 5). The fusion genes involved in such alteration encode proteins normally implicated in the control of hematopoiesis, cell growth, and differentiation. The most frequent targets of translocation in acute leukemia involve genes that function as transcription factors, such as core binding factor (CBF), retinoic acid receptor alpha (RARA), members of the *HOX* gene family, and transcriptional modulatory proteins (4, 7).

Among transcription factor genes, CBF complex is of particular interest because of its frequent alterations found in many subtypes of AML (6, 7). CBF is a family of heterodimeric transcription factors that are crucial for the regulation of hematopoietic system. This family comprises one of three different DNA-binding α

subunits and a single common β subunit (6). The α subunit contains a highly conserved, runt domain that mediates DNA-binding ability and heterodimerization with β subunit (6). The β subunit does not directly contact DNA but enhances the DNA-binding ability (8). The α and β subunit of CBF complex are encoded by *AML1* (*acute myeloid leukemia 1*) gene on chromosome 21q22 and *CBFβ* gene on chromosome 16q22, respectively (9). Thus far, more than a dozen different chromosomal translocations in AML cases that involved *AML1* are known. Relevant examples of these molecular lesions include t(8;21), t(12;21), and t(3;21) which generate AML1-ETO, TEL-AML1, and AML1-EVI1 fusion proteins, respectively (6, 10). The chromosomal translocation t(8;21)(q22;q22) is one of the most common chromosomal aberrations in AML-M2 subtype (11). The translocation fuses the *AML1* gene on 21q22 with the *ETO* gene on 8q22 resulting in the production of a chimeric *AML-ETO* mRNA. AML patients with t(8;21) translocation had a better overall prognosis than other AML subtypes (12).

Although *AML1* gene was deregulated in AML cases mostly through chromosome translocations as stated above, *non-translocation* mechanisms of *AML1* deregulation, in spite of its rare occurrence, have recently been described such as point mutations in patients with AML and gene amplifications in patients with acute lymphoid leukemia (ALL) (13). Minimally differentiated AML (M0 AML), a rare subtype of AML associated with poor prognosis, has been reported to have a high frequency of *AML1* mutations ranging from 12% to 50% (22-23). *AML1* mutations were infrequently detected in other subtypes of AML (M1-M5, M7) and had never been reported in M6 cases. Most mutations involved the amino (N)-terminal region of AML1, particularly the runt domain, which is encoded by exons 3-5, and was rarely found in the carboxy (C)-terminal region (13, 14). AML patients with *AML1* mutation had a significantly worse prognosis than those without mutation (15).

Leukemia arises from a mutated clone of hematopoietic stem cells or progenitors cells in the bone marrow. For leukemia to arise, at least 2 classes of gene mutations must exist according to the model of AML/MDS/MPD recently proposed by Gilliland et al in 2002 (16). Class I mutations are exemplified by constitutively

activated tyrosine kinases and their downstream effectors, such as *BCR/ABL*, *RAS* and *FLT3*. The aberrations of genes within this class confer a proliferative and/or survival advantage to hematopoietic progenitor cells (4, 16). On the other hand, Class II mutations cause the loss of function of transcription factors through chromosomal translocations such as *AML1/ETO*, *CBFß/SMMHC*, or *PML/RARα*, as well as point mutations in *AML1* and C/EBPα, and interfere with normal hematopoietic differentiation process (16, 17). Several lines of evidence support this concept whereby *AML1* gene rearrangements could be shown to cause loss of function of hematopoietic transcription factors but were not by themselves sufficient to cause frank AML in animal models (18).

At present, no data exists in Thailand and neighboring Southeast Asian countries with regard to the incidence and type of *AML1* gene abnormalities and their significance in adult AML patients. Moreover, the cooperative events in AML patients with *AML1* deregulations are presently unknown. At present, no molecular techniques to detect *AML1* gene alterations have been available in Thailand. Thus the aim of study was to characterize two main mechanisms of *AML1* gene abnormalities, i.e. translocation and point mutation, in a large cohort of Thai adult *de novo* AML patients. The correlation between AML genotype and patients' clinical characteristics, karyotypes and immunophenotypes was also explored. To search for cooperative events in AML, I also determined whether the mutation of the *FLT3* receptor tyrosine kinase (Class I mutation) could be an additional mutation associated with leukemic transformation in AML patients harboring the *AML1-ETO* fusion gene or *AML1* mutation (Class II mutation).

CHAPTER II
OBJECTIVES

1. To develop the molecular methods for the detection of genetic abnormalities involving *AML1* gene, particularly, *AML1-ETO* gene rearrangement and *AML1* point mutation.
2. To determine the incidence of *AML1-ETO* gene rearrangement and type of *AML1* point mutation in Thai adult *de novo* AML population.
3. To explore if *AML1-ETO* gene rearrangement and *AML1* point mutation have any unique association with patients' clinical features, karyotypes, and immunophenotypes.
4. To determine if *FLT3* tyrosine kinase receptor mutation coexists in patients with *AML1* gene abnormalities.

CHAPTER III
LITERATURE REVIEW

1. Acute myeloid leukemia (AML)

1.1 Definition and cause of AML

AML is a cancer of the bone marrow and blood characterized by a rapid uncontrolled growth of myeloid progenitor cells (19). AML is a major hematopoietic malignancy in the world. This disease is characterized by the proliferation of a malignant clone, resulting in the production of blasts and other immature granulocytes, monocytes, erythrocytes, and/or megakaryocytes (20).

1.2 Clinical features

The clinical signs and symptoms of AML are variable and nonspecific, but these are usually the results of leukemic infiltration of the bone marrow which leads to cytopenia. Typically, patients present with pallor, hemorrhage, infections and fever due to the decrease in red cells, platelets, and white cells, respectively. Additionally, some AML patients have leukemic infiltration of various tissues, including the liver, spleen, skin, lymph nodes, bone, gingiva, and central nervous system. Hyperleukocytosis can lead to symptoms of leukostasis, such as ocular and cerebrovascular dysfunction (21).

1.3 Etiology

Although the cause of AML in humans is incompletely understood, a variety of hereditary and environmental factors appear to play an etiologic role. The pathogenesis of AML involves complex interactions between host susceptibility, chromosomal damage secondary to physical or chemical exposure, and the incorporation of genetic information transmitted virally into susceptible progenitor cells.

1.3.1 Radiation

The ionizing radiation has also been associated with leukemogenesis. Much of the epidemiologic evidence is observation after human exposures to radiation, nuclear explosion, therapeutic or diagnostic radiation, and from occupational radiation. Patients who were treated with external-beam irradiation for ankylosing spondylitis, menorrhagia, and with radioactive phosphorus (^{32}P) for polycythemia vera have an increased incidence of leukemogenesis (22).

1.3.2 Chemical agents

Exposure to alkylating agents such as melphalan or nitrogen mustard is associated with the development of therapy-related AML (t-AML), after a latency of 5 to 7 years (22). This disease is characterized by pancytopenia, trilineage myelodysplasia, and complex cytogenetic abnormalities, most often involving loss of part or all of chromosomes 5 or 7 (22). Another subtype of t-AML has been observed in patients treated for certain hematologic and solid neoplasms with the epipodophyllotoxin drugs. Organic solvents such as benzene and other petroleum products have also been associated with a higher risk of developing AML (23).

1.3.3 Viruses

A number of viruses are known to cause acute leukemia. The Epstein-Barr virus (EBV) genome is present in the majority of Burkitt leukemia/lymphoma cells. Adult T-cell leukemia is closely linked to infection by a leukemogenic virus, human T-cell lymphotropic virus type I (HTLV-I). However, most people who have antibodies to this virus usually do not exhibit any evidence of malignancy.

1.3.4 Heredity and genetics

People with certain blood disorders such as myelodysplasia or some genetic disorders are known to have a higher risk of developing leukemia. In identical twins, if one twin has leukemia, the other is also at risk for leukemia, often before 8 years of age. The disease usually develops in the second twin within a year of the first twin's diagnosis. Chromosomal instability in several autosomal dominant conditions including Fanconi's anemia, ataxia telangiectasia, neurofibromatosis, and Bloom's syndrome are associated with acute leukemia. Recently, germline mutations in the

AML1 gene are known to be associated with an increased risk of the development of AML. Additionally, congenital immunodeficiency disorders including infantile X-linked agammaglobulinemia and Down's syndrome have also been associated with an increased incidence of AML (24, 25).

1.4 Diagnosis

The diagnosis of AML is based upon a careful examination of the peripheral blood and bone marrow. The bone marrow is replaced by myeloblasts, which can be further characterized using cytochemistry, immunophenotyping and cytogenetics. The diagnosis of AML requires the identification of more than 20% leukemic blasts in the bone marrow. The primary diagnosis of AML is based on the morphologic identification of leukemic myeloblasts in peripheral blood and bone marrow stained with Wright–Giemsa. This distinction is based on the morphology of the blast cells and their reactivity with histochemical stains, including myeloperoxidase, sudan black, the nonspecific esterases α-naphthylacetate and naphthylbutyrate. Cytogenetic or direct molecular genetic methods have become an essential part of the routine diagnosis of patients with AML. Moreover, several cytogenetic abnormalities can be used to identify subgroups of patients with distinct clinical features and therapeutic responses. This combination of morphologic, immunologic, and genetically based diagnostic approaches not only makes it possible to modify therapy according to the sensitivity of biologically defined subtypes, but also provides unique markers to monitor the patient response to therapy.

1.5 Classification of AML

There are several classification systems which separate AML patients into various subtypes. The most commonly used method for AML classification was developed by the French–American–British (FAB) group (2). According to the FAB criteria, AML can be divided into eight distinct subtypes (Table 1). The FAB criteria are based on morphologic features of leukemic cells as determined by the degree of differentiation and cell lineages (Table 2). Immunologic methods have also recently been incorporated into the diagnostic criteria of the FAB subgroups. The FAB subtypes are composed of three types with predominantly granulocytic differentiation

(M1, M2, and M3), two types with at least 20% monocytic precursors (M4 and M5), one type with a high proportion of erythroblasts (M6), and a more recently recognized and rarely occurring variant with the predominance of megakaryoblasts (M7). In addition, the FAB group described a form of AML with minimal myeloid differentiation, designated as M0, which cannot be diagnosed solely on morphologic or cytochemical groups but requires the additional use of immunohistochemical staining. M0 cases express myeloid antigens on the blast cell surface but lack myeloperoxidase reactivity.

Table 1 The FAB classification of AML (2).

Table 1 The FAB classification of AML (2).

FAB subtype	Common name	Frequency (%)
MO	Acute myeloblastic leukemia with minimal differentiation	2-3
M1	Acute myeloblastic leukemia without maturation	15-20
M2	Acute myeloblastic leukemia with maturation	25-30
M3	Acute promyelocytic leukemia	8-15
M4 **M4Eo**	Acute myelomonocytic leukemia Acute myelomonocytic leukemia with abnormal eosinophils	20-25 5
M5	Acute monocytic leukemia	10
M6	Acute erythroleukemia	5
M7	Acute megakaryoblastic leukemia	1-2

Table 2 Morphological and cytochemical features of AML (1).

FAB classification	Morphologic and cytochemical features
M0	Large, agranular myeloblasts; stained negative for myeloperoxidase and Sudan black
M1	Large, poorly differentiated myeloblasts represent 90% or more of the nonerythroid cells; at least 3% of the myeloblasts stained positive for myeloperoxidase
M2	Between 30% and 89% of the nonerythroid cells are myeloblasts having abundant cytoplasm with moderate to many granules; Auer rods often visible
M3	Leukemia cells usually contain heavy azurophilic granulation; nuclear size varies greatly; nuclei are often bilobed or kidney shaped; some cells contain bundles of Auer rods; leukemia cells stained strongly positive for myeloperoxidase
M4	Myeloblasts consitute over 30% of the nonerythroid cells, but total granulocytic precursors do not exceed 80%; monocytic cells account for >20% of the nonerythroid cells; nonspecific esterase and chloroacetate stains are often positive; Auer rods may be present.
M5	Monoblasts, promonocytes, or monocytes constitute 80% or more of the nonerythroid cells; in one subtype (M5A), 80% or more of all the monocytic cells are monoblasts; in the well-differentiated subtype (M5B), less than 80% are monoblasts; a-naphthyl acetate positivity is extinguished by NaF
M6	Greater than 50% of the nucleated marrow cells are erythroid; erythroblasts are usually strongly PAS positive; myeloblasts represent 30% or more of the nonerythroid cells
M7	Large and small megakaryoblasts with high nuclear/cytoplasm ratio; cytoplasm is pale and agranular; standard cytochemical stains are not definitive; platelet peroxidase and platelet-specific antibodies are often positive

Table 3 shows the classification of AML according to World Health Organization (WHO) criteria. The basic principle of the WHO system is that the classification of hematopoietic and lymphoid neoplasms should utilize not only morphologic findings but also all available information, including genetic, immunophenotypic, biologic, and clinical features to define specific disease entities (26, 27).

In the WHO classification, AML can be classified into four subgroups. Three distinct subgroups are as follow: (i) AML with recurrent genetic abnormalities, (ii) AML with multilineage dysplasia, and (iii) AML and MDS, therapy related. AML with recurrent genetic abnormalities subgroup recognizes four well-defined recurring genetic abnormalities, including cases of patients with t(15;17), t(8;21), inv(16) or t(16;16), and abnormalities of 11q23. AML patients associated with t(15;17), t(8;21), inv(16) or t(16;16) have distinctive clinical findings and a favorable response to appropriate therapy. The fourth subgroup is "AML, not otherwised categorized" which contains cases that do not satisfy the criteria for any of these subgroups, or for which no genetic data can be obtained. This group is morphology-based and reflects the old FAB classification with a very few modifications.

Table 3 The WHO classification of AML (27).

Acute myeloid leukemia with recurrent genetic abnormalities
Acute myeloid leukemia with t(8;21)(q22;q22), (*AML1/ETO*)
Acute myeloid leukemia with abnormal bone marrow eosinophils and inv(16)(p13q22) or t(16;16)(p13;q22), (*CBFβ/MYH11*)
Acute promyelocytic leukemia with t(15;17)(q22;q12), (*PML/RARα*) and variants
Acute myeloid leukemia with 11q23 (*MLL*) abnormalities
Acute myeloid leukemia with multilineage dysplasia
Following MDS or MDS/MPD
Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in 2 or more myeloid lineages
Acute myeloid leukemia and myelodysplastic syndromes, therapy related
Alkylating agent/radiation–related type
Topoisomerase II inhibitor–related type (some may be lymphoid)
Others
Acute myeloid leukemia, not otherwise categorized
Classified as:
Acute myeloid leukemia, minimally differentiated
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic/acute monocytic leukemia
Acute erythroid leukemia (erythroid/myeloid and pure erythroleukemia)
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma

1.6 Prognostic factor

AML patients can be separated into three different prognotic groups based on their response to induction treatment, relapse risk, and overall survival (2). Furthermore, assessment for multidrug resistance and cytogenetic characteristics of the leukemic blasts are also important. The Southwestern Oncology Group (SWOG) and Medical Research Council (MRC) classify AML patients into three distinct prognostic groups including favorable, intermediate or unfavorable subgroup based on karyotypes (Table 4) (19).

Typically, patients with favorable risk cytogenetics have either t(15;17), t(8;21), or inv(16) or molecular evidence of these abnormalities. These mutations are more frequent in younger patients who have high complete remission rate (more than 85%) and a relatively low risk of relapse (30 to 40%). At the other end of the spectrum is the unfavorable prognostic subgroup, which includes approximately 15% of the cases among patients who are 15 to 60 years of age. The unfavorable cases are defined by the presence of leukemic blasts with complex abnormalities involving more than two chromosomes, monosomies of chromosome 5 or 7, deletion of the long arm of 5 (del5q), or abnormalities of the long arm of chromosome 3. These abnormalities are more frequent in older patients and in patients with secondary AML. Between these two groups are patients who are characterized as having intermediate or standard risk. The leukemic blasts of these patients have either a normal karyotype or cytogenetic abnormalities that are not included in the definition of the other subgroups (3, 19).

Table 4 SWOG and MRC criteria classification of AML prognosis factors (19).

Clinical outcome	SWOG Criteria	MRC criteria: As for SWOG, except:
Favorable	t(15;17) with any other abnormality inv(16)/t(16;16)/del(16q) with any other abnormality t(8;21) without del(9q) or complex karyotype	t(8;21) with any other abnormality
Intermediate	+8, -Y, +6, del(12p) normal karyotype	abn 11q23 del(9q), del(7q) without other abnormalities Complex karyotypes (≥ 3 abnormalities, but < 5 abnormalities) All abnormalities of unknown prognostic significance
Unfavorable	-5/del(5q), -7/del(7q), t(8;21) with del(9q) or complex karyotype inv(3q), abn 11q23, 20q, 21q, del(9q), t(6;9) t(9;22), abn 17p, Complex karyotypes (≥ 3 abnormalities)	Complex karyotypes (≥ 5 abnormalities)
Unknown	All other clonal chromosomal aberrations with fewer than 3 abnormalities	

SWOG: Southwestern Oncology Group
MRC: Medical Research Council;
abn: abnormalities of

1.7 Cytogenetic abnormalities of AML

Chromosomal abnormalities can be detected in the majority of AML. These abnormalities include gain or loss of a whole chromosome or loss of the long (q) or short (p) arm of chromosome (28). Furthermore, a variety of structural rearrangements (translocation, inversion, or insertion) were also found (29). Cytogenetic characterization has become the strongest predictor of both responses to therapy and remission duration. Cytogenetic data have been used to map a chromosomal breakpoint at a molecular level, allowing the use of probes for fluorescence in situ hybridization (FISH) and of primers for reverse transcriptase polymerase chain reaction (RT-PCR) methods for the detection of tumor cells (30).

1.8 Molecular pathogenesis and transcription factor in AML

Recent advances in genetics have shown that not only chromosomal abnormalities but also molecular alterations are associated with the leukemogenesis of AML (31, 32). The most common targets of AML-associated chromosomal translocation are genes that encode DNA-binding transcription factors or the regulatory components of transcriptional complexes (29). These transcription factors are frequently conserved and are important in normal haemotopoiesis. The common balanced reciprocal translocations associated with AML are a fusion of two transcription factors (Figure 1). Transcription factors involved in human leukemia are composed of core binding factor (CBF), retinoic acid receptor alpha (RARα), homeobox (HOX) family members, and members of the ETS family of transcription factors (Table 5) (4, 17). Transformation in each of these cases appears to result from the generation of fusion proteins that interfere in a dominant manner function of the wild-type protein (20). The study of these specific molecular genetic lesions has provided critical insights into the pathogenesis of AML and has already helped to identify subgroups for therapeutic purposes (16).

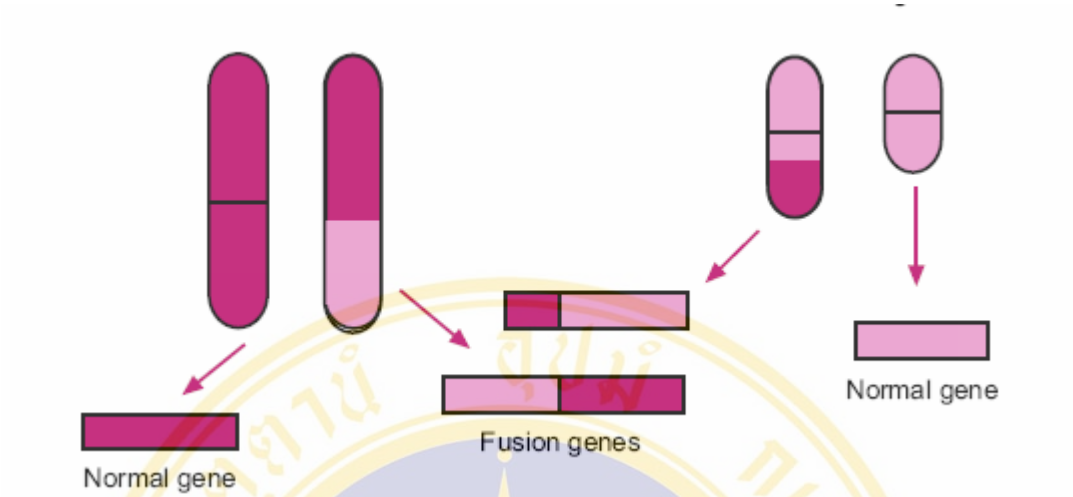


Figure 1 Balanced reciprocal translocations in human leukemia may give rise to both forward and reciprocal fusion transcript, and both normal alleles for each gene may also be expressed (17).

Table 5 Transcription factor fusions in human leukemia (17).

Translocation	Fusion gene
A. Core binding factor (CBF)	
t(8;21)(q22;q22)	*AML1/ETO*
t(3;21)(q26;q22)	*AML1/EVI1*
inv(16)(p11;q22)	*CBFβ/SMMHC*
t(12;21)(p13;q22)	*TEL/AML1*
B. Retinoic acid receptor α (RARα)	
t(15;17)(q22;q11)	*PML/RARa*
t(5;17)(q31;q11)	*NPM/RARa*
t(11;17)(p13;q11)	*PLZF/RARa*
C. HOX family members	
t(7;11)(p15;p15)	*NUP98/HOXA9*
t(2;11)(q31;p15)	*NUP98/HOXD13*
t(12;13)(p13;q12)	*TEL/CDX2*
D. ETS family members	
t(12;22)(p13;q11)	*MNI/TEL*
t(16;21)(p11;q22)	*TLS/ERG*

1.9 Cooperativity of leukemia genes in AML

AML has been proposed to occur as a consequence of the collaboration between at least two broad classes of mutations (Figure 2) (16, 17, 33). Class I mutation is exemplified by activating mutations in tyrosine kinases such as *BCR–ABL, TEL–PDGFRB* or *FLT3*, or their downstream effectors, such as *RAS* mutations. These mutations confer proliferative and/or survival advantage to hematopoietic progenitor cells but has minimal effects on their differentiation programs. In contrast, Class II mutation resulting in loss of function of hematopoietic transcription factors that are important for normal hematopoietic differentiation including *AML1-ETO*, *CBFPβ-SMMSC*, *PML-RARα* and *NUP98/HOXA9* fusion genes. Moreover, point mutations in hematopoietic transcription factors such as *AML1* and *C/EBPα* are also classified as Class II mutation (17). These mutations also confer an immortalization phenotype but are not sufficient to cause AML. If the mutations that confer proliferative and/or survival advantage are expressed alone, they result in a myeloproliferative disorder (MPD) with leukocytosis and normal differentiation (16). Conversely, when mutations that impair a normal differentiation expressed alone, these mutations may have a phenotype most like myelodysplastic syndrome (MDS) (16). Together, these two mutations would result in the AML phenotype characterized by a proliferative and/or survival advantage of hematopoietic progenitors and by impaired hematopoietic differentiation. For example, targeting the prolifereative and survival pathway with *FLT3* inhibitors or RAS inhibits such as farnesyltransferase inhibitors may have a therapeutic benefit. Alternatively, agents that relieve the block in differentiation of leukemic blasts have been shown to have a therapeutic benefit such as all-trans-retinoic acid (*ATRA*) that is now the standard treatment for acute promyelocytic leukemia. One class of compounds currently that is under investigation is histone deacetylase (*HDAC*) inhibitors (33).

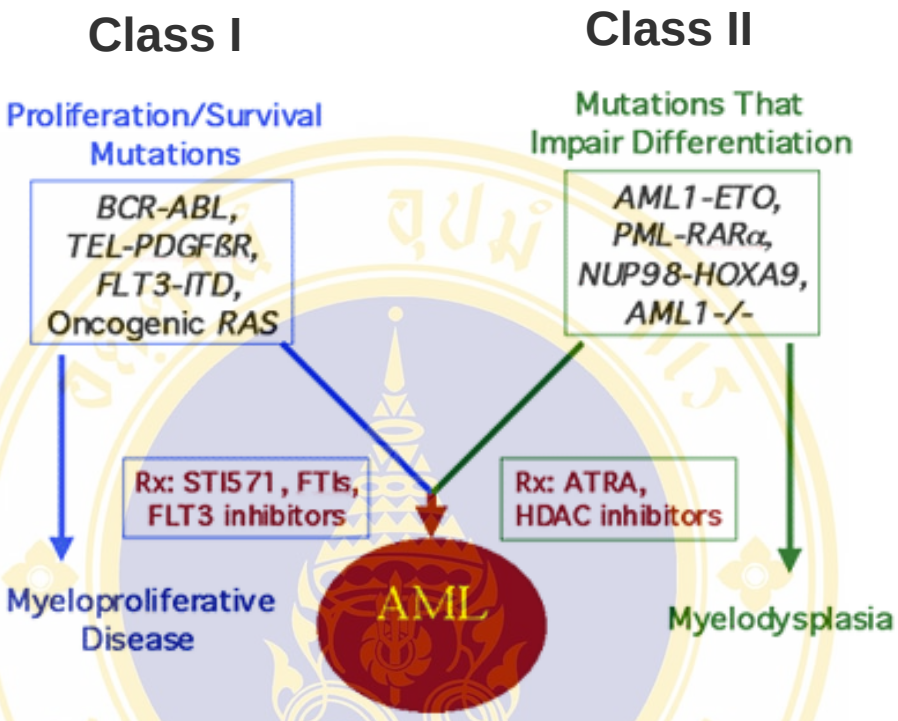


Figure 2 Cooperative mutation between Class I and II mutations. Class I mutations are gene rearrangements and mutations that confer a proliferative and/or survival advantage to leukaemia cells, and Class II mutations are those that impair haematopoietic differentiation in AML (16).

2. Core binding factor (CBF) in AML

CBF was first identified based on its ability to interact with the enhancer core sites of polymavirus or moloney murine leukemia virus and is also known as polyoma enhancer binding protein 2 (PEBP2) (9). Chromosomal abnormalities involving subunits of CBF are common in AML. Many of these translocations are involved with transcription factors or transcriptional co-activators that are important for normal hematopoietic development. Of these, the most extensively studied are the *AML1-ETO* and *CBFβ-SMMHC* fusion gene (33).

2.1 Structure of CBF complex

CBF is a class of hematopoietic transcription factors that are crucial for the regulation of hematopoietic system. The CBF complexes comprise one of three different α subunits and a single common β subunit (Figure 3) (17). The α subunit is located in nucleus but β subunit is in the cytoplasm. The α subunit and β subunit of CBF complex are encoded by *AML1* (also known as *RUNX1*) and *CBFβ* gene, respectively. The α subunit mediates DNA-binding ability and heterodimerization with β subunit. The β subunit does not directly contact DNA but enhances the DNA-binding ability of the α subunit and protects it from proteolysis. CBF complex regulates the expression of a large number of hamatopoiesis regulated gene.

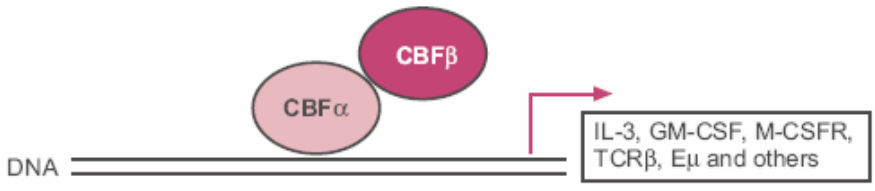


Figure 3 Structure of the core binding complex (CBF). The core binding factor (CBF) consists of two subunits, α and β subunits which are encoded by the *AML1* and *CBFβ* genes, respectively.

2.2 Biochemical functions of CBF complex

Recent studies indicate that CBF complex plays an important role in normal hematopoiesis. The normal hematopoiesis development consists of two distinct waves. The first wave is primitive hematopoiesis which emerges in the yolk sac at embryonic day 7.5. The second wave is definitive hematopoiesis, which occurs on embryonic day 9.5 (Figure 4). CBF complex is required for the generation of hematopoietic stem cells from the endothelium of the great vessels and promotes the differentiation of mature cells from the hematopoietic progenitor cells (6, 33).

Under normal conditions, CBF complex binds to a consensus DNA core motif known as the runt domain binding element (TGt/cGGT). This consensus sequence is present in the transcriptional regulatory regions of genes that are essential for myelopoiesis. These include the genes encoding for interleukin-3 (IL-3) (34), granulocyte–macrophagecolony stimulating factor (GM-CSF) (35), CSF1 receptor, myeloperoxidase, neutrophil elastase, granzyme B, and subunits of the T- and B-cell antigen receptor. Therefore, impaired CBF interaction is likely to interfere with normal myelopoiesis (6).

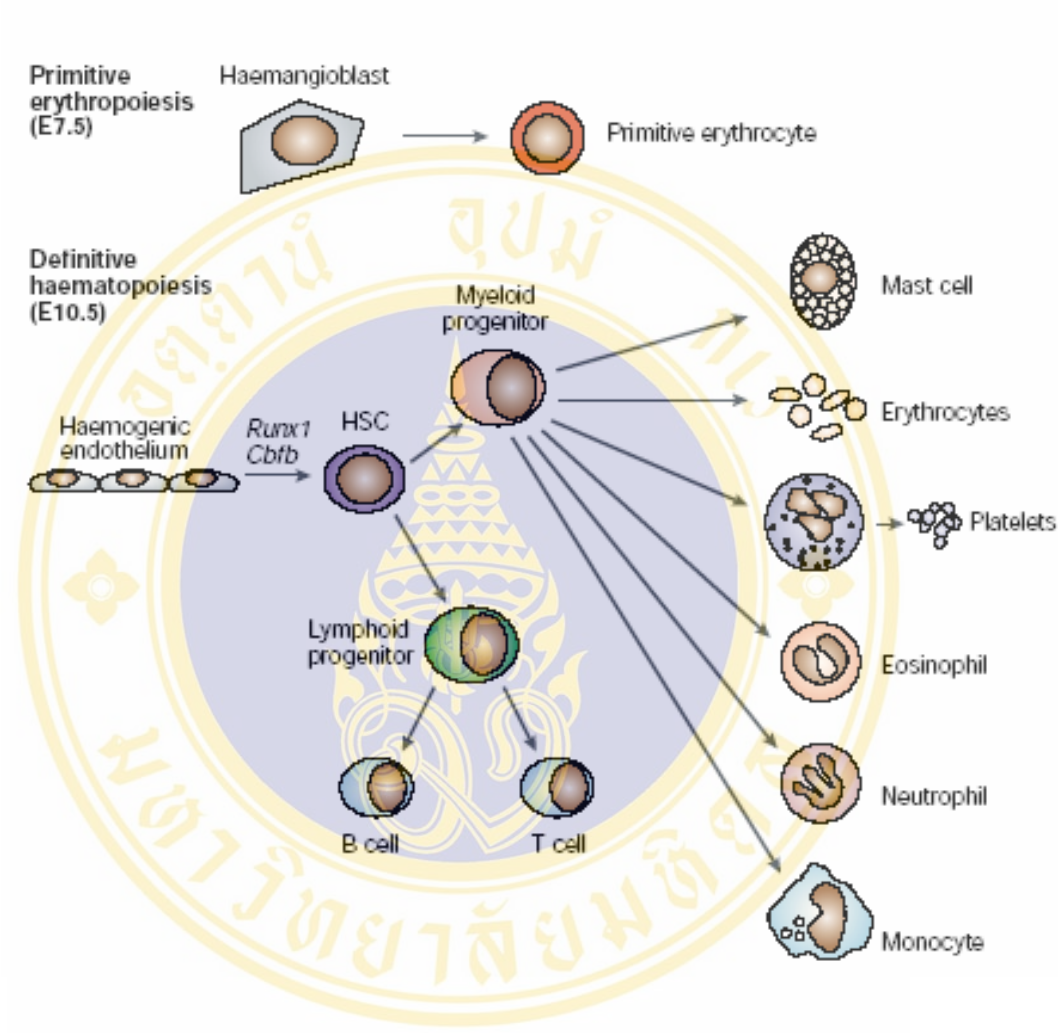


Figure 4 Haematopoiesis in the fetus and in the adult. *AML1* and core-binding factor-β (*CBFβ*) are not required for the development of primitive erythrocytes, but are required for the development of the haematopoietic stem cell (HSC) to different blood cell lineages (33).

3. Structure of *AML1* gene and protein

Acute myeloid leukemia 1 (AML1) gene is also called *Runx1* (Runt-related protein), *PEBP2αB* (polyoma virus enhancer-binding protein2αB), and *CBFα* (core binding factor α) (6). The *AML1* was initially cloned as the target of t(8;21) in patients with AML (5). This gene is located on chromosome 21q22 and spans 260 kb of the genome. The *AML1* gene consists of nine exons (exons 1-6, 7A, 7B, and 8) and two promoters followed by the distinct initiation codon in exon 1 and exon 3 (14).

AML1 protein possesses at least three alternatively spliced isoforms, which include AML1a, AML1b, and AML1c (36). These three isoforms are distinctly expressed in hematopoietic cells. Nucleotide sequence analysis of the cDNA clones showed that the predicted AML1 proteins could be classified into two types, designated AML1b (453 amino acids) and AML1c (480 amino acids), with different N-terminal regions (Figure 5). AML1b and AMLlc contain the same large C-terminal region, which is rich in proline, serine, and threonine (36, 37). This C-terminal region has been suggested to be a transcriptional activation domain. The runt domain is fully conserved among the three species. Exons 1 and 3 contain the 5'-untranslated region and the ATG initiation codon. AML1c arises from the use of an internal splice acceptor site in exon 1, while AMLla and AML1b begin at exon 3, probably due to utilization of an alternative promoter (36). The runt domain is encoded by a part of exon 3, exon 4, and exon 5. Exons 7A and 8 contain the TAA and TGA termination codons, respectively, and the entire 3'-untranslated region. As a result of alternative splicing, AML1a contains exon 7A. On the other hand, AML1b and AML1c contain exons 7B and 8, resulting in proteins with a larger C-terminal (36). The three patterns of alternative splicing are schematically shown in Figure 5. In AML1b and AML1c, carboxy (C)-terminal to the runt domain has a region that contains sequences of defined biochemical functions that is absent in AML1a. AML1b is largely restricted to hematopoietic cells, including B and T lymphoid cells, granulocytic and monocytic cells, megakaryocytes, and CD34+ marrow cells. AML1b is also presented in erythroid precursors during embryogenesis, but not in adult marrow (38).

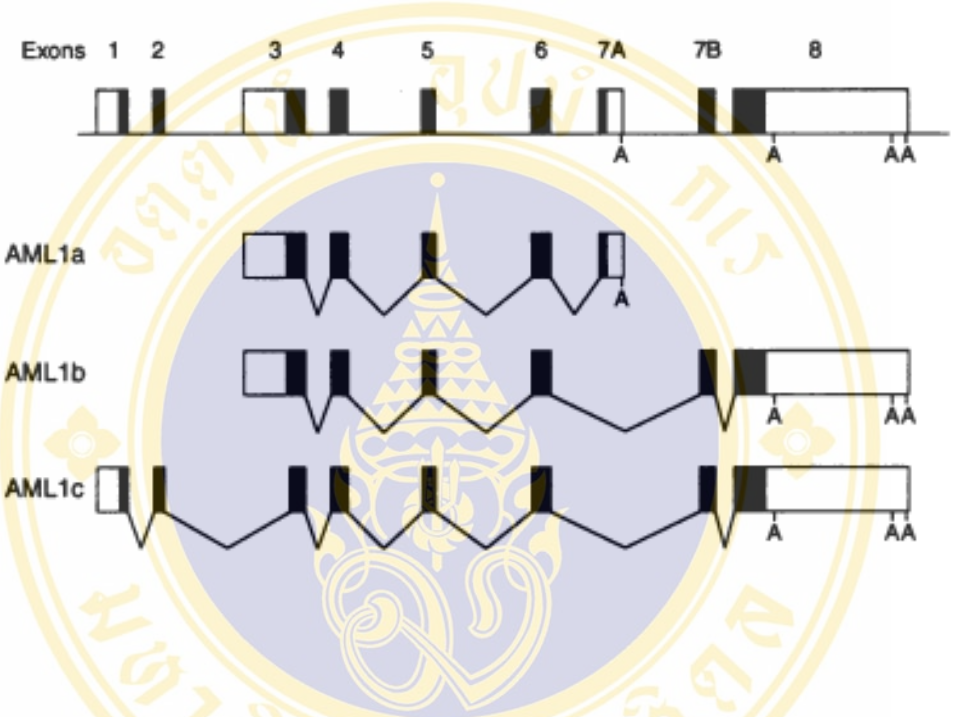


Figure 5 Schematic representation of alternative splicing of the AML1 transcripts. A schematic representation of the exons is shown at the top. Solid boxes represent the coding regions, and open boxes represent the 5'- and 3'- untranslated regions. Positions of polyadenylation signals are shown by A (36).

The structure of AML1 protein is shown in Figure 6. AML1 contains the most highly conserved feature at the runt domain which is responsible for both DNA-binding ability and heterodimerization with the β subunit of the CBF complex (38, 39). The consensus sequence for DNA binding is PyGPyGGT and named the PEBP2 sequence. The PEBP2 sequence is found in transcriptional cis-elements of AML1 tareget genes, to which CBF complex binds and initiates transcription (6). The AML1 also contains transactivation and transcriptional repression domains which lead AML1 to function as an activator or repressor of gene transcription, respectively (6, 8).

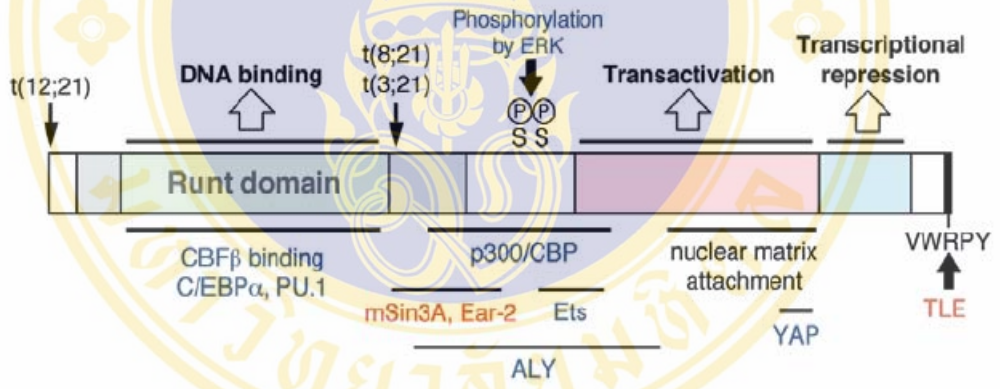


Figure 6 Functional anatomy of AML1 protein. DNA binding and heterodimerization with CBFβ are mediated by the runt domain. Arrows indicate breakpoints in t(8;21), t(3;21), and t(12;21). The locations of other sequences for transcription (Red), transcriptional repression (Blue), and nuclear matrix attachment are shown. The phosphorylation is mediated by ERK site. P300/CBP, ALY, YAP are binding regions for coactivators. The mSin 3A, Ear-2, and TLE are binding regions for corepressors (6).

3.1 Structure of the runt domain

The runt domain is encoded by a part of exon 3-5, which contains 128 amino acids that span 50-178 of amino acids sequences (Figure 7) (40). The cDNA nucleotide and amino acids sequences of the runt domain of *AML1* gene are shown in Figure 8. The runt domain belongs to the immunoglobulin-domain superfamily of transcription factors (33). DNA binding by the runt domain is mediated by loops and β-strands at one end of the immunoglobulin β-barrel. The CBFβ subunit increases the DNA-binding affinity of the runt domain without inducing large conformational changes in the runt domain (9-11).

AML1 makes a contact with the DNA at the consensus sequence 5'-TGTGGTT-3' using three loop-containing regions, including β3-L3, β9-L9, and β12-L12 (37). Three arginines (R80, R174, and R177) recognize three guanines of DNA. K83 interacts with the sugar-phosphate backbone of the DNA. The backbone amide of G143 and V170 interact with the phosphate backbone in the minor and major grooves, respectively (33). Amino acids in the runt domain that are mutated in patients with familial platelet disorder with a propensity to develop acute myeloid leukaemia (FPD/AML), AML M0 and MDS are R80A, W79C, R142A, G138D, D171A, R174A, and R177A (33, 37, 41). Most of these mutations disrupt amino acids that directly contact the DNA, whereas the G138D and W79C mutations could either indirectly disrupt the DNA contacts of neighboring amino acids, or perturb the runt domain fold (37).

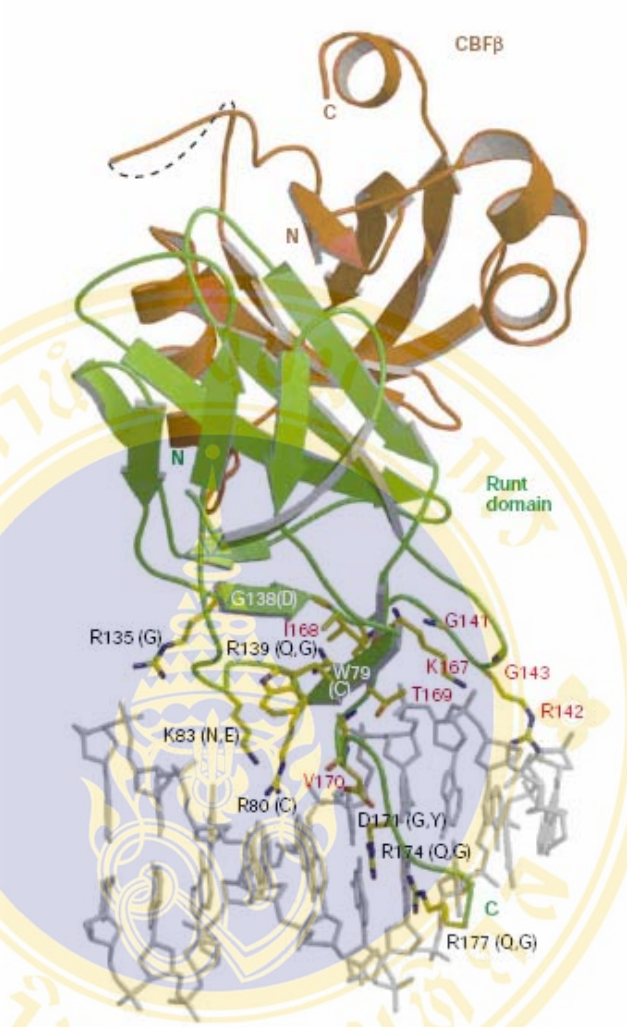


Figure 7 Structure of the runt domain. The runt domain (green) belongs to the immunoglobulin-domain superfamily of transcription factors. DNA binding by the runt domain is mediated by loops and β-strands at one end of the immunoglobulin β-barrel. CBFβ (brown), which has a novel structure contacts neither the DNA, nor amino acids in the runt domain that are directly involved in DNA binding. Amino acids in the runt domain that directly contact DNA are indicated by black or red text. Amino acids in the runt domain that are mutated in patients with FPD/AML, AML M0 and MDS are indicated in black or white text, with the mutations shown in parentheses (33).

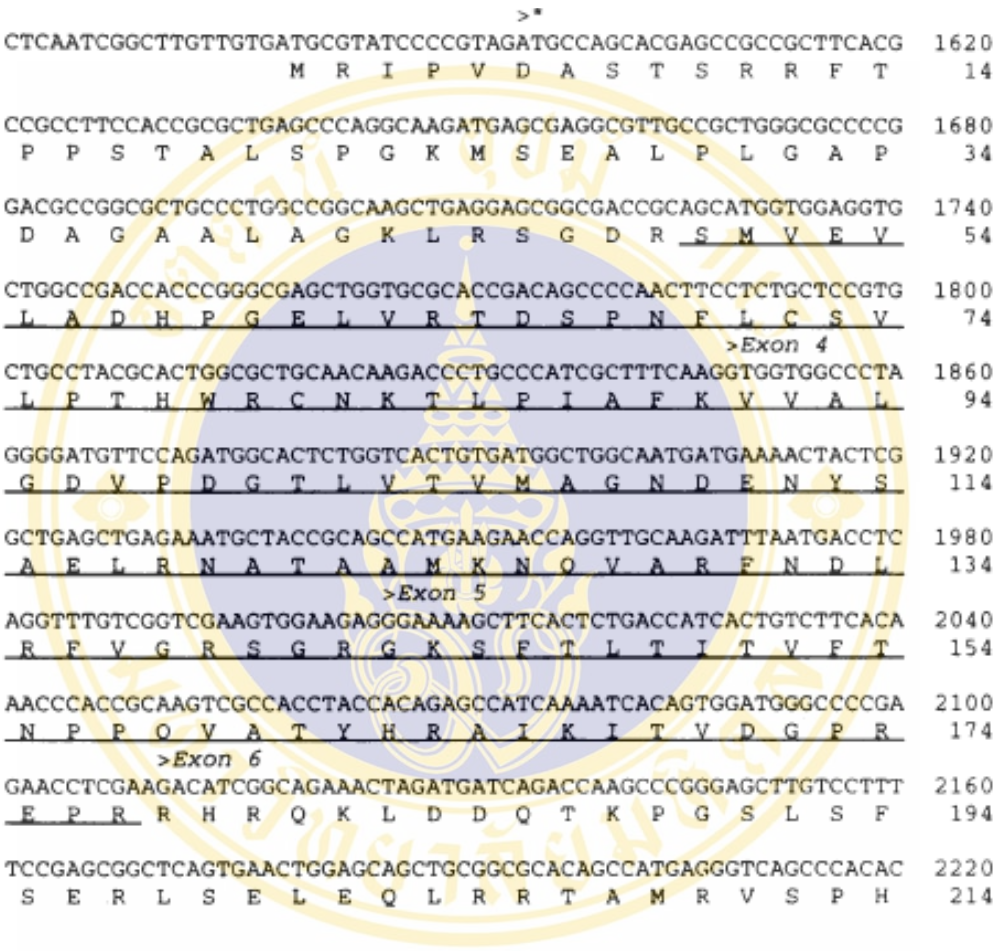


Figure 8 Nucleotide and amino acid sequences of the runt domain of AML1b. The runt domain is underlined. Boundaries of exons are indicated by arrowheads above nucleotide sequences, at the beginning of each exon. Alternative splice sites are also indicated with an asterisk. The sequence data reported here have been deposited in the NCBI sequence databases under the accession numbers D43968 (36).

3.2 Different partner proteins augment transcriptional activation or repression by AML1

Although AML1 functions as an organizing protein that facilitates assembly of transcriptional activation complex (Figure 9). Table 6 shows the partner proteins that interact with AML1 protein (8). The PEBP2 sequence is often adjacent to the binding sites for other DNA-binding proteins, including Ets, Myb, and C/EBPα. All of these factors are known to cooperate with AML1 to activate gene transcription. In addition to these *cis*-interactions, AML1 associates with transcriptional cofactors including p300 and CREB-binding protein (CBP), which facilitate transcription by acetylating histones and rendering the promoter structures more accessible. Other coactivators that interact with AML1 include Yeast-associated protein (YAP) and ALY, the latter is a protein that activates the TCRα promoter by facilitating assembly of LEF-1, Ets, and AML1 (42). At these transcriptional regulatory sequences, AML1/CBFβ appears to function as a transcriptional organizer that recruits other factors into a nucleoprotein complex that stimulates lineage-restricted transcription. Although originally identified as a transcriptional activator, AML1/CBFβ contains at least three domains that can contribute to the repression of transcription. When interacting with transducin-like enhancer of split-1 (*TLE1*), a mammalian homologue of the *Drosophila* co-repressor Groucho, it mediates transcriptional repression. Interaction between AML1 and the co-repressor mSin3A also appears to be necessary for this transcriptional repression. Subsequent binding to a co-repressor, such as *TLE-1* or mSin3A would convert AML1 to a repressor. The transcriptional repressor of AML1 depends on the target gene and the cellular milieu. AML1 acts as a transcriptional repressor in NIH3T3 cells and CD4 in thymocytes (41, 42). The mechanism that converts AML1 from an activator to a repressor are not yet fully understood. One possibility is posttranslational modification, including phosphorylation by extracellular signal-regulated kinase which stimulates transcriptional activity of AML1 (41).

Table 6 Partner proteins that interact with AML1 (42).

Partner protein	Reported function	Function of AML1 partner complex
Myb	Transcription factor	Co-activation of TCRδ enhancer
Ets	Transcription factor	Co-activation of transcription
C/EBP	Transcription factor	Co-activation of M-CSF receptor promoter
P300/CBP	Transcriptional co-activator	Myeloid cell differentiation
ALY	Transcriptional co-activator	Co-activation of TCRα enhancer
YAP	Transcriptional co-activator	Transcription co-activation
TEL1	Co-repressor	Contributes to Runx1/AML1-mediated repression
mSin3A	Co-repressor	Necessary for AML1 transcriptional repression

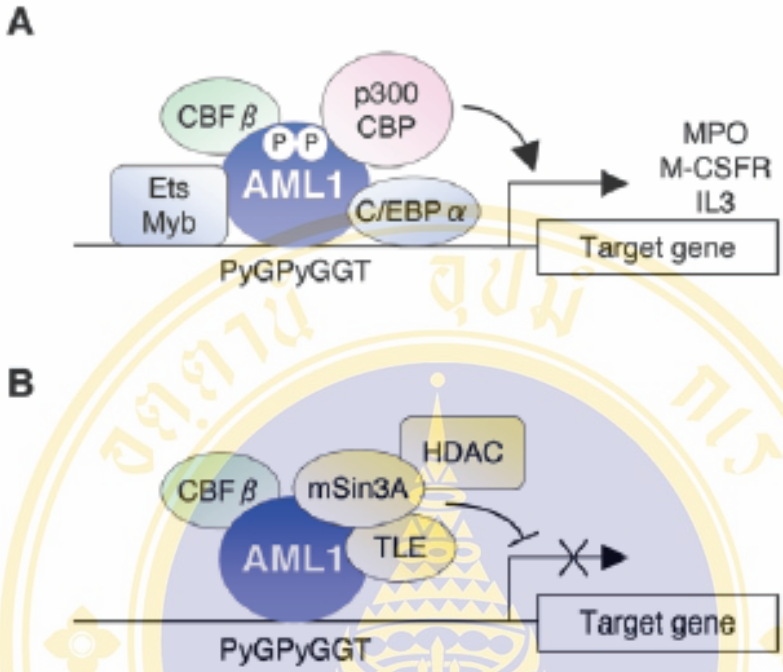


Figure 9 Transcriptional complex formation of AML1. AML1 mediates transcriptional activation (A) or repression (B) upon recruitment of non-DNA-binding coactivators (p300/CBP) or corepressors (mSin3A, HDAC, and TLE) (6).

3.3 *AML1* gene alteration in AML patients

AML1 is deregulated in AML patients through different mechanisms including chromosomal translocation, point mutation, and gene amplification (13). Thus far, more than a dozen different chromosomal translocations in AML cases that involved *AML1* are known. The t(8;21) which generate *AML1-ETO* was the most common chromosomal translocation associated with AML (43). This fusion protein has a common functional property as a dominant negative inhibitor for the normal AML1 allele. In addition to t(8;21), *AML1* has been shown to be involved in other translocations in other type of leukemia such as t(12;21)(*TEL-AML1*), which occurs in approximately 25% of cases of pediatric B-cell acute lymphoblastic leukemia and confers favorable outcome (44, 45). The t(3;21) (*AML1-Evi1*), was found in a few cases of secondary AML and blast crisis of chronic myeloid leukemia (Figure 10) (46).

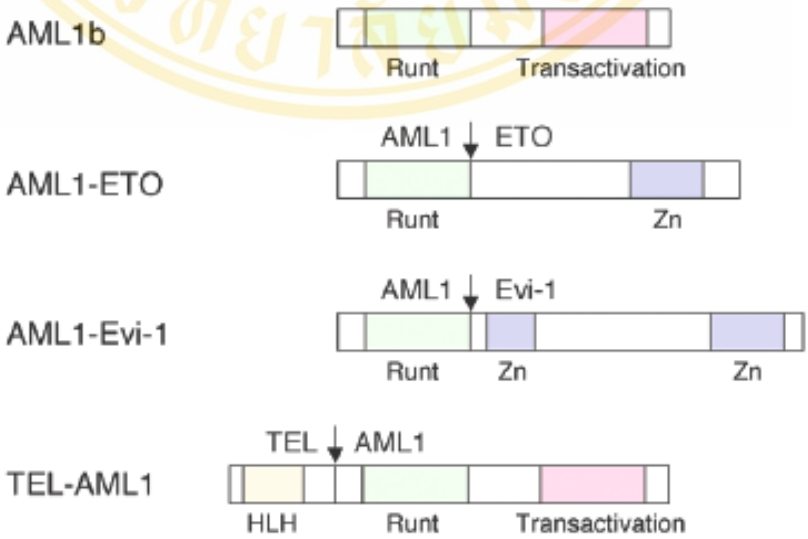


Figure 10 Structure of fusion proteins generated in AML1-related leukemia. Arrows indicate breakpoints in the fusion genes (6).

4. *AML1-ETO* fusion gene in acute myeloid leukemia

In 1973, Rowley firstly described a balanced translocation between chromosomes 8 and 21, t(8;21)(q22;q22) (5). This translocation fuses the *AML1* gene on chromosome 21q22 and the *ETO* gene on chromosome 8q22, resulting in the production of the AML1-ETO fusion mRNA and protein (5, 29). The AML1-ETO fusion consists of 177 amino acids N-terminal of AML1 fused inframe with amino acids 30-604 of ETO (12, 47, 48). Thus this fusion protein retains the runt domain that binds DNA and interacts with CBFβ but deletes the C-terminal activation domain of AML1 (Figure 11) (12, 49, 50).

ETO is the mammalian homolog of the Drosophila gene *nervy*, and is a member of a small gene family that includes two other members, *MTGR1* and *MTG16* (51). The *ETO* locates on chromosome 8q22 and contains 13 exons distributed over 87 kb of genomic DNA (52-54). The ETO protein contains four regions (52, 55) including an N-terminal domain with homology to transcription-activating factors (TAF), a hydrophobic heptad repeat (HHR), a small region with homology to the ETO group of proteins referred to as the Nervy homology region 3 (NHR3), and a C-terminal domain that contains two Zn-finger motifs (51). The expression of ETO is normally restricted to cells in the nervous system and a subset of CD34 hematopoietic progenitors (54). ETO has been detected in the nucleus of leukemic cells and in the nucleus and cytoplasm of neurons (56).

Recent data suggest that ETO normally functions as a regulator of transcription. This protein directly interacts with the nuclear co-repressors NcoR and mSin 3A, and recruits an active histone deacetylase (51, 57). These observations suggest that ETO may function as an adapter protein within a nuclear co-repressor complex.

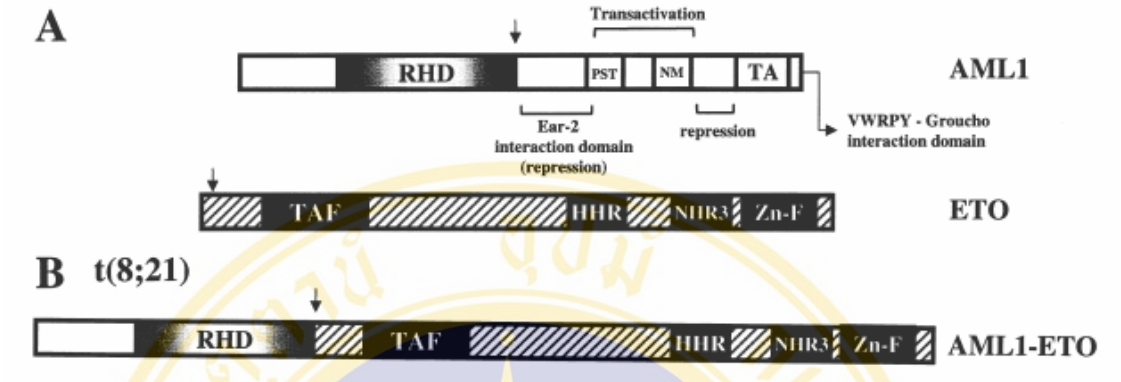


Figure 11 Structure of AML1-ETO fusion protein. (A) represents AML1 protein. (B) The structure of the t(8;21)-encoded AML1-ETO product is illustrated. Chromosome breakpoints are indicated by vertical arrow (58).

4.1 Incidence of *AML1-ETO* in acute myeloid leukemia

The t(8;21)(q22;q22) is the most frequent translocation found in AML. By conventional karyotypic analysis and RT-PCR, the t(8;21) (*AML1-ETO*) translocation is detected in about 6-20% of AML cases and in 18-40% of AML-M2 (12). *AML1-ETO* has also been found, at lower frequencies, in AML-M1 and M4, and in rare cases of myelodysplastic and myeloproliferative syndromes (58).

4.2 The mechanism of *AML1-ETO* fusion in leukemogenesis

The *AML1-ETO* fusion gene can lead to the disruption the differentiation process of hematopoiesis system (59). As a consequence of the t(8;21), AML1 sequences from C-terminal to the runt domain are replaced by the majority of the coding region of ETO (Figure 12) (6). Thus, the resultant fusion protein entirely lacks the transcriptional activation domain of AML1. As predicted by this structure, AML1-ETO is no longer capable of interacting with the transcriptional coactivators p300 or CBP, but instead acquires many partners of the ETO binding protein (60). Consistent with the above model, AML1-ETO directly represses AML1-mediated transcriptional activation in transient transcription assays (61). This activity depends on both the runt domain of AML1 and the HHR and zinc fingers of ETO. Transcriptional repression

requires direct DNA binding by the AML1-ETO fusion protein through the core enhancer sequence and is mediated through the recruitment of the nuclear corepressor complex by ETO (58, 60). AML1-ETO has also been demonstrated to repress the transcriptional activity of C/EBPα and a subset of Ets proteins (62). This activity is independent of the core enhancer sequence, but instead is mediated by the direct binding of AML1-ETO to either C/EBPα or the Ets family members, resulting in changes in chromatin structure by the tethered histone deacetylase corepressors. The transcriptional repression by AML1-ETO can be partially reversed by inhibitors of histone deacetylase, consistent with the critical role of this enzymatic activity in mediating the transcriptional effects of AML1-ETO (63).

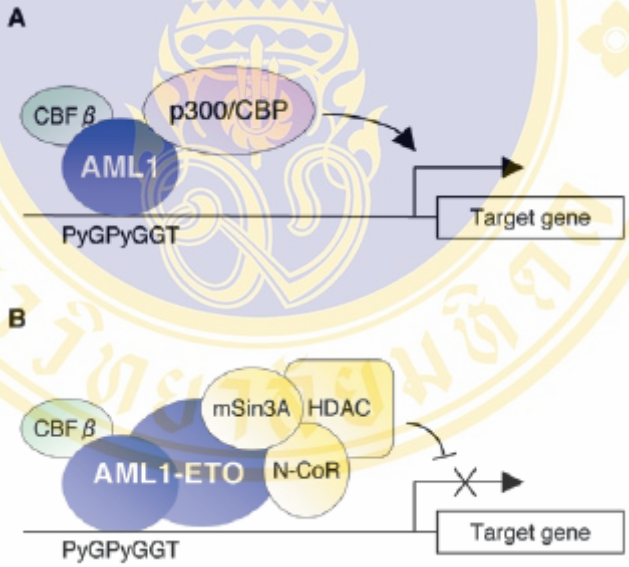


Figure 12 Transcriptional complex formation by AML1. (A) AML1 binds to the consensus DNA sequence (PEBP2 sequence) present in the *ci*elements of target genes. The PEBP2 sequence is often adjacent to inding sites for other DNA-binding proteins, including Ets, Myb, and C/EBPα. AML1 regulates gene expression in cooperation with these lineage-specific transcription factors. AML1 mediates transcriptional activation (A) or repression (B) upon recruitment of non-DNA-binding coactivators (p300/CBP) or corepressors (mSin3A, HDAC, and TLE) (6).

4.3 *In vivo* and *in vitro* study of *AML1-ETO*

Experiments using a variety of different cell lines have been performed in an attempt to define the biological activity of the *AML1-ETO* fusion gene. The *AML1-ETO* knock-in mice lacks fetal hematopoiesis (61). *AML1-ETO* acts as a dominant inhibitor of normal CBF activity. Animals heterozygous for an *AML1-ETO* knock-in allele displayed a phenotype similar to that of *AML1* or *CBFβ* knock-out mice, resulting in an early death in embryonic life (embryonic day 13.5) and intracranial bleeding (Figure 13A). The dysplastic hematopoietic progenitor cells within the fetal livers of the knock in mice could be readily established as immortalized cell lines (Figure 13B) (61, 64). Importantly, expression of *AML1-ETO* does not completely block the ability of these cells to differentiate, but instead impairs maturation.

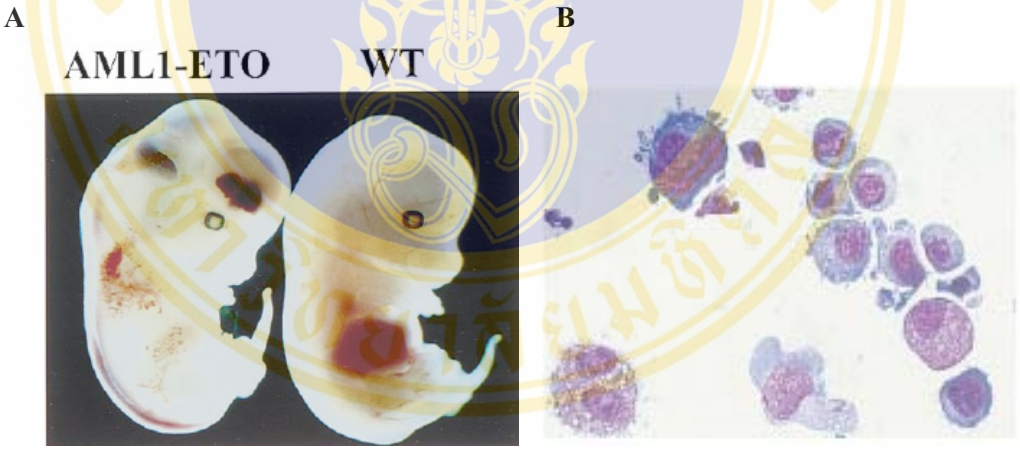


Figure 13 Phenotype and morphology of *AML1-ETO* knock-in and wild-type (WT) embryos. *AML1-ETO* knock-in embryos were identical in size to wild-type littermates, but were easily identifiable by the presence of fetal liver pallor and massive hemorrhages within the ventricles of the CNS and the soft tissues of the back (A). Numerous hypergranular myeloid cells are seen with frequent abnormal binucleated and trinucleated cells (B) (61).

4.4 Cooperation of *AML1-ETO* with other abnormalities

One of the most interesting results emerged from these experiments was that expression of *AML1-ETO* by itself was insufficient to generate a full leukemic phenotype (12, 65). Similarly, adult bone marrow cells infected with an *AML1-ETO* expressing retrovirus failed to induce leukemia when transplanted into irradiated syngeneic mice. The development of leukemia, therefore, appears to require additional genetic mutations that are able to cooperate with the signals generated through *AML1-ETO*. The analysis of *AML1-ETO*-containing human leukemias may provide some clues to the nature of the secondary mutations. For instance, almost 50% of *AML1-ETO* expressing leukemias have been found to involve with loss of one of the sex chromosomes (12). In addition, over 30% of these cases have deletions on the long arm of chromosome 9 (47, 58, 66). Although the specific genes targeted by these genetic changes are at present unknown. The recurrent loss of these loci raises the possibility that they harbor tumor suppressor genes, whose loss cooperates with *AML1-ETO* to induce a full leukemic phenotype. More recently, activating mutations in the *C-KIT* of *FLT3* receptor tyrosine kinase have been identified in a significant percentage of patients *AML1-ETO* leukemia (18, 67). Although these data are derived from only a small cohort of cases, they suggest that constitutively activated growth factor signals may cooperate with *AML1-ETO* to induce transformation (35, 68, 69).

4.5 Clinical significance and immunophenotypic analysis of AML patients with *AML1-ETO* fusion

AML patients whose leukemic blasts contain *AML1-ETO* fusion gene constitute a favorable prognostic group, characterized by high remission rates and long-term disease-free survival when treated with chemotherapy that includes cytarabine (19, 70, 71).

The immunophenotypic analysis of AML patients with *AML1-ETO* fusion gene usually discloses CD13, CD33, CD34, and CD56 expression (70, 72). Interestingly, positive staining for the B-cell associated marker CD19 has been reported in a significant co-expression with *AML1-ETO* fusion gene (73). The *AML1-ETO* positive AML patients had high remission rates and prolonged disease free survival, with a more favorable overall prognosis than that of other AML subsets (58).

Thus, identification of *AML1-ETO* at diagnosis is important for prognostic stratification.

4.6 Detection of *AML1-ETO* fusion gene

Several methods have currently been used to detect the *AML1-ETO* rearrangement. While the conventional analysis was karyotypic analysis (74), Fluorescent in situ hybridization (FISH), southern blot analyses (75), RT-PCR, and real-time PCR (76, 77) are techniques that allow specific detection of two genes fused at the breakpoint of chromosomal translocations (78, 79). Recently, RT-PCR has been used successfully as a tool for identifying fusion genes (79). Previous studies indicate that the result of karyotype analysis and RT-PCR are not completely identical (74, 80). Importantly, RT-PCR can detect the fusion transcript in AML patients whose leukemic cells lack t(8;21) cytogenetically (74). RT-PCR has now become a routine method in the Western countries whereby the results are rapidly available, resulting in early decision making particularly in AML patients with unsuccessful karyotypic analysis (80).

5. *AML1* mutation in AML

Although t(8;21) which generated *AML1-ETO* fusion gene frequently found in the pathogenesis of AML, point mutation of the *AML1* gene has also been hypothesized to play an important role (13, 14).

5.1 Incidence of *AML1* mutation in AML

All *AML1* mutation screening studies covered exons 3–5, and most included intron sequences flanking the exons. A few studies screened exons 1 and 2 as well, and half of the studies did not cover exons 6–8 which encoding the C-terminal moiety where the transactivation domain spanning amino acids 291–371 is located (14). All types of human leukemia, except chronic lymphocytic leukemia (CLL) (81), have been extensively investigated. In addition, a significant number of cell lines, including those established from solid tumors, as well as healthy volunteers were also included in the mutation screening (14). The frequency of *AML1* mutation in *de novo* AML is low (<5%), and has been detected at a substantially higher frequency in a specific

subtype of AML, i.e., poorly differentiated AML M0 (12-33%) (14, 82). Most point mutation identified in AML patients involve the amino acids at N-terminal region of this transcription factor, especially in the runt domain, which is encoded by exons 3-5. In AML patients, *AML1* mutation is rarely found in the C-terminal region (15, 83).

5.2 Type of *AML1* mutation

Germline and acquired mutations of *AML1* were reported in patients with hematological malignancies (13). Germline mutation of the *AML1* has been found in very rare cases of familial platelet disorder with predisposition to acute myelogeneous leukemia (FPD/AML) (84). In all cases, mutations were mono-allelic which resulted in haploinsufficiency and susceptibility to leukemogenesis (84). In patients who carry *AML1* germline mutations, leukemia does not develop until the acquisition of the second mutation later in life (16, 33). Recently, acquired somatic mutations of *AML1* gene was also reported in rare cases of AML and MDS (15, 71, 85). In hematological malignancies, haploinsufficiency of *AML1* may also have a pathogenic role and mono-allelic alteration of *AML1* was also found (41).

5.3 *AML1* mutation and leukemogenesis

AML1 acts as a key regulator of hematopoietic genes that plays an important role in myeloid differentiation (8). The deregulation of this gene may contribute to leukemogenesis in different ways.

Functional consequences of bi-allelic *AML1* point mutations are the absence of DNA binding properties and/or the abolishment of transactivation of the targeted genes which involved in myeloid differentiation and maturation (8, 11, 86). Moreover, some of the mutations act in a dominant negative manner and prevent a normal function of the remaining wild-type *AML1,* in the case of mono-allelic alteration (15). The studies that cotransfected with *AML1* missense mutation and wild-type *AML1* have confirmed this hypothesis and demonstrated the abolishment of transactivation of the M-CSF receptor promoter (15). *AML1* mutation could be involved in leukemogenesis through other mechanisms. Indeed, many of the *AML1* alterations lead to the loss of the C-terminal domain which is crucial for interaction with the TLE,

groucho corepressor gene (15, 87). In all of those cases, the mutated protein could not act to repress transactivation.

5.4 Correlation between *AML1* mutation and other findings

On the other hand, mutated cases had a significantly higher leukocyte count, a higher percent of bone marrow blasts, lower CD33 expression, higher HLA DR expression and more frequent heavy chain immunoglobulin (Ig H) or T cell receptor (TCR) gene rearrangement (13, 88). AML patients who expressed *AML1* mutations had a significantly worse prognosis than those without the mutations (15).

5.5 Cooperation between *AML1* mutation and other genetic abnormalities

FLT3 mutation and trisomy 21 are thought to be the second hit in MDS/AML with *AML1* mutations (89-91). High frequency of chromosome 7 loss suggests that inactivation of the unknown gene on chromosome 7 also plays a synergistic role with *AML1* mutations (92). Furthermore, to induce leukemogenesis, three to five genetic alterations are considered to be required based on current evidence (14). Therefore, a number of genetic alterations must be identified to fully understand the leukemogenesis.

5.6 SSCP analysis for the screening of *AML1* mutation

Recently, SSCP has been extensively applied in biomedical research with various adaptation and refinements. SSCP is the most suitable sensitive technique for detecting only one base change (93-95). Other common techniques include targeted restriction fragment length polymorphism (RFLP) analysis, heteroduplex analysis and its derivatives, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (13). All these techniques have their advantages and disadvantages in terms of sensitivity, reproducibility, optimization, cost and requirements for equipment (95).

The SSCP technique is a method capable of identifying most sequence variations in a single strand of DNA, typically between 150 and 250 nucleotides in length. Under nondenaturing conditions, a single stranded DNA is adopt a conformation (presumably dependent on internal base-pairing between short segments

by foldback) that is uniquely depended on its sequence composition (94). This conformation is usually different if even a single base is changed. Most conformations seem to alter the physical configuration or size sufficiently that, even though the variant sequence has the same charge, the configuration-to-charge (size-to-charge) ratio is different enough to be detectable as a mobility difference upon electrophoresis through a retarding matrix such as acrylamide gel (95).

6. *AML1* amplification in acute leukemia

Recent analysis of the *AML1* locus has shown that high-level gene amplification of *AML1* can be detected in some cases of human leukemia (96, 97). Those cases primarily constitute pediatric ALL cases, some of whom have been confirmed to have an increase in *AML1* transcripts. These observations support the hypothesis that gain of function or increased dosage of *AML1* can contribute to leukemogenesis (41).

7. *FLT3* mutation

Fms-like tyrosine kinase 3 (*FLT3*) is a member of the class I receptor tyrosine kinase subfamily, including *C-FMS, C-KIT* and *PDGFR*. *FLT3* is expressed in immature hematopoietic cells and plays an important role in the proliferation of hematopoietic progenitors in combination with other growth factors (98). The internal tandem duplications (ITDs) in the juxtamembrane (JM) domain of *FLT3* are demonstrated in approximate 20% of AML. In addition, substitution mutations in the *FLT3* second tyrosine kinase (TK2) domain at D835 (*FLT3*-D835) have recently been reported in approximately 7% of patients with AML. Both the *FLT3*-ITD and *FLT3*-D835 mutations result in constitutive activation of the receptor that confers IL-3-independent growth in murine hematopoietic cell lines 32Dcl3 and Ba/F3 with ligand-independent dimerization, autophosphorylation and activation of downstream pathways such as STAT5 and MAP kinase (16). These *FLT3* mutations confer an increased relapse rate in several studies (16).

8. Possible targets for therapy of CBF leukemia

The understanding of the molecular pathogenesis of CBF leukemias provides several potential for therapeutic intervention. It might be possible to identify small molecules that specifically inhibit the translocation fusion proteins without affecting the function of native CBF (99). Such small molecules might reverse the block in differentiation in hematopoietic cells that are mediated by leukemogenic fusion proteins, resulting in differentiation and subsequent apoptosis of cells. Finally, small molecules that would stabilize DNA binding in cases of hemizygous inactivating mutations might have therapeutic efficacy. For example, in a patient with loss of function of a single *AML1* allele, molecules that stabilize or enhance transcription that is mediated by the gene product of the residual allele might allow for normal hematopoietic differentiation (98). Collectively, the analysis of CBF has provided important for the normal homeostatic mechanisms that govern hematopoietic development, and how disruption of CBF function can contribute to the leukemogenesis. These insights should, in turn, provide a foundation for the development of new molecularly targeted therapies for CBF leukemias. Recently several studies showed that the specific inhibitors of tyrosine kinases, including *FLT3* and *c-KIT*, might also prove useful in a subset of CBF leukemias (33).

CHAPTER IV
MATERIALS AND METHODS

1. Materials

1.1 Leukemic and normal samples

Archived consecutive samples of newly diagnosed adult *de novo* AML patients at the Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University were available for complete analysis in this study. The study is a part of a large Leukemia Project funded by Mahidol University and approved by the Ethical Committee, Faculty of Medicine Siriraj Hospital. Approximately 2-4 mL of bone marrow (BM) or 5-10 mL peripheral blood (PB) samples were collected from each consented patient. AML was diagnosed by the standard morphological, cytochemical, and immunophenotypic methods according to the FAB Classification (100).

Four hundred and fourteen patients from year 2000-2004 were used for *AML1* point mutation detection whereas 108 patients from year 2004-2005 were included in *AML1-ETO* fusion gene analysis.

1.2 Oligonucleotide primers

Figures 14, 15, and 16 show the locations of the three primer pairs designed to amplify the runt domain of *AML1*. Nucleotide sequences, numbers, and product sizes after amplifications with all primers are listed in Table 3.

PCR of *AML1-ETO* fusion gene was amplified using three primers, AE_F1, AE_R1 and AE_ F2 (Figure 17). The sequences of these primers were shown in Table 5. Figure 16 shows the diagram of the primers used to amplify *AML1-ETO*. The first round PCR amplification was performed using AE_F1 and AE_R1 primers. The AE_F1 primer was *AML1* sense primer responsible for the nucleotides 1163-1184 based on the nomenclature of Miyoshi et al, 1991 (55). The AE_R1 was the *ETO* antisense primer that binds to nucleotide 100-120 downstream to the *AML1-ETO* breakpoint. The second semi-nested PCR was performed using the same AE_R1 but

with an internal *AML1* primer, AE_F2. The AE_F2 was the nucleotides 1201-1220 based on the nomenclature of Miyoshi et al, 1991 (55). The AE_F1-AE_R1 pair of primers and the AE_F2-AE_R1 generated 261 and 223 bp fragment, respectively. Additionally, one pair of primer for the amplification of β-actin cDNA (BA_F1 and BA_R1) was included to assess the integrity of RNA.

1.3 Monoclonal antibodies

For flow cytometric analysis, a cocktail of monoclonal antibodies (BD Biosciences, CA, USA) were used as listed in Table 7.

1.4 Other materials

Chemicals, instruments, enzymes, reagents and others are listed in the appendix.

Table 7 Relevant antigens in immunophenotyping of acute leukemias.

Designation	Cell type	Antigen
CD2	T-cells, most NK cells	Erythrocyte-rosette receptor
CD3	Surface expression on mature T-cells, cytoplasmic	Associated with the T cell receptor, mediates signal expression in immature T cells transduction
CD5	Thymocytes, mature T cells, subpopulation of B cells	Linked to T cell proliferation
CD7	T-cells, NK-cells, subpopulation of immature myeloid cells	40 kDa protein
CD10	c-ALL, lymphatic precursor cells, neutrophils subset of mature B cells	Common acute leukemia antigen (CALLA), neutral endopeptidase
CD11c	Monocytes, neutrophils, NK-cells, subpopulation of B-cells	Adhesion molecule, gp 150/95
CD13	Myeloid cells	Aminopeptidase N
CD14	Monocytes and neutrophils	LPS receptor
CD16	NK-cells, neutrophils, subpopulation of monocytes	Low-affinity Fc-receptor for IgG
CD19	Precursor B-cells, B-cells	Bridge for surface immunoglobulin signal
CD20	Subpopulation of precursor B-cells, B-cells	Ion channel, protein kinase C substrate
CD22	Surface expression on B-cells, cytoplasmic expression in precursor B-cells	Related to neutral cell adhesion molecule, bridge for surface immunoglobulin signal
CD33	Monocytes, myeloid precursor cells, weak expression on	67 kDa glycoprotein neutrophils
CD34	Myeloid and lymphoid precursor cells	105-120kDa glycoprotein
CD41a	Platelets, megakaryocytes	Glycoprotein Iib/IIIa, fibrinogen receptor
CD45	All leukocytes	T200 antigen, protein-tyrosine-phosphatase
CD56	NK-cells	N-CAM
CD64	Monocytes, macrophages	High-affinity receptor for IgG
CD117	Myeloid precursors cells	C-kit, stem cell factor receptor
HLA-DR	B-lymphocytes, activated T-lymphocytes, monocytes	Part of the MHC II complex precursor cells
Glycophorin A (GlyA)	Erythrocytes, erythroblasts and erythroid precursor cells	Sialinic acid-rich polypeptide
Nuclear terminal deoxynucleotidyl transferase (TdT)	Nuclear expression in lymphoid precursor cells	Terminal deoxynucleotidyl transferase
Myeloperoxidase (MPO)	Lysosomal expression in neutrophils and monocytes,	Myeloperoxidase including immature myeloid cells

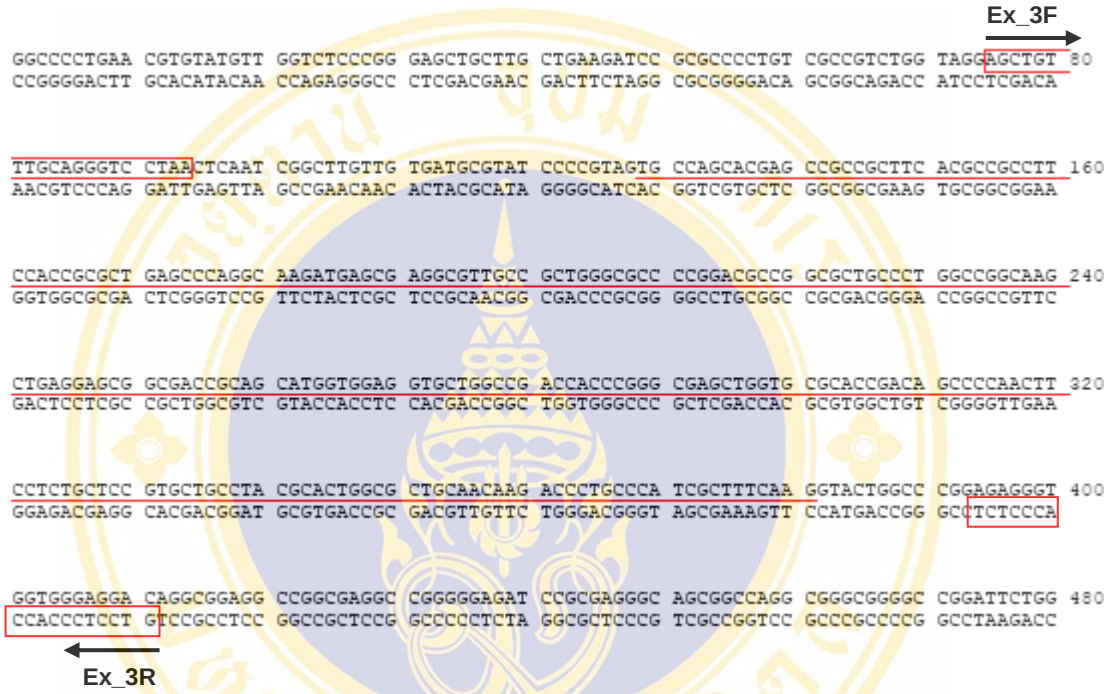


Figure 14 Nucleotide sequences for the PCR amplification of exon 3 of *AML1* gene. Primers are indicated in blocks. Underlined were sequences of exons. Arrows indicate 5' to 3' direction of the primers (primers Ex_3F and Ex_3R). Complete sequence of the *AML1* gene (accession number D43968) was retrieved from GenBank database.

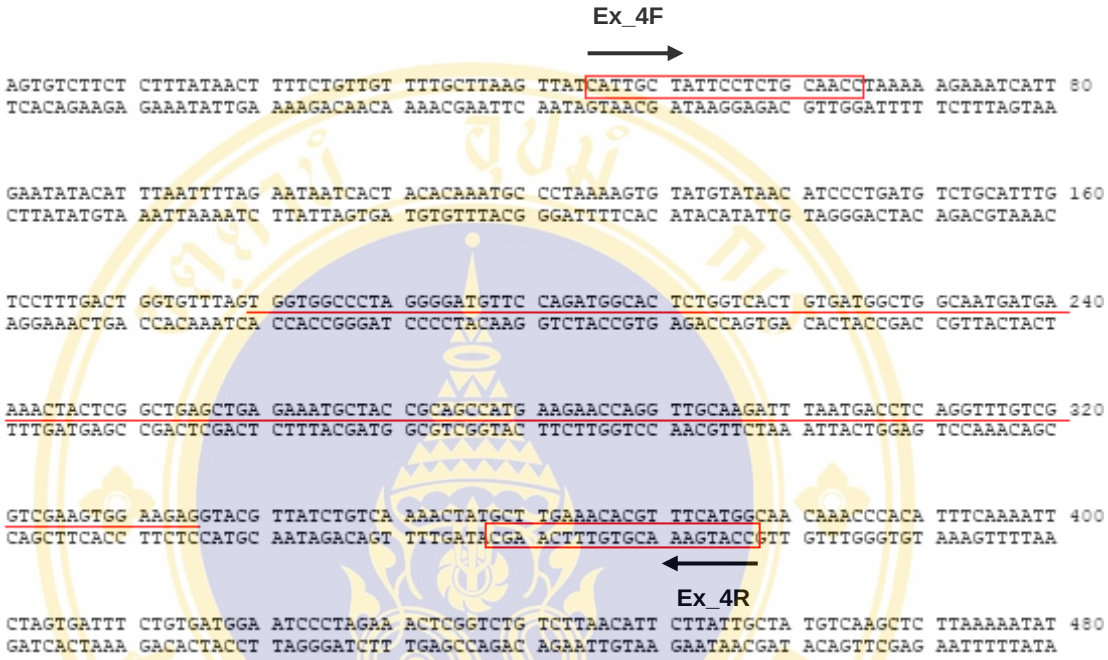


Figure 15 Nucleotide sequences for the PCR amplification of exon 4 of *AML1* gene. Primers are indicated in blocks. Underlined are sequences of exons. Arrows indicate 5' to 3' direction of the primers (primers Ex_4F and Ex_4R). Complete sequences of the *AML1* gene (accession number D43968) was retrieved from GenBank database.

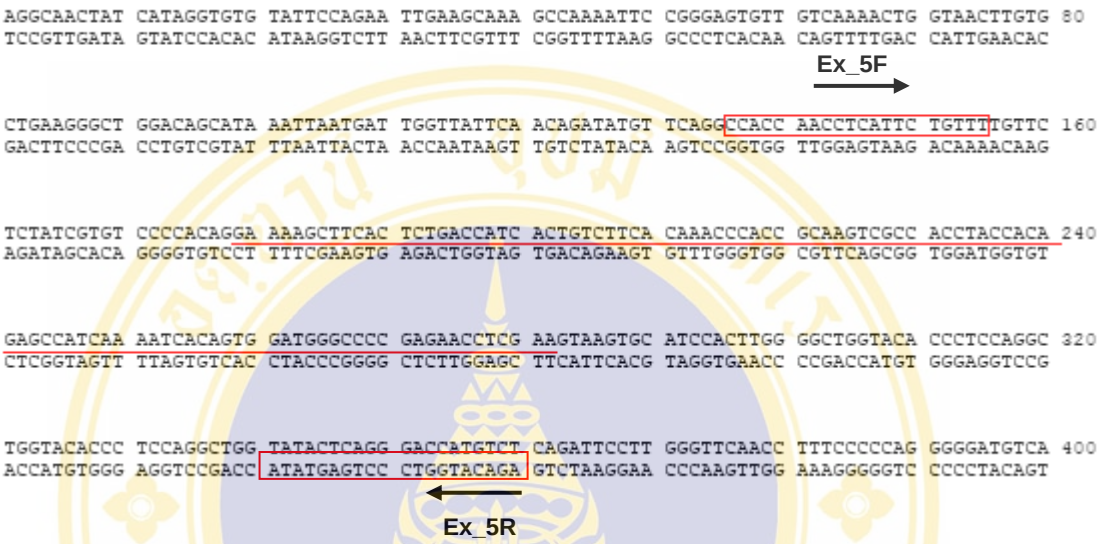


Figure 16 Nucleotide sequences for the PCR amplification of exon 5 of *AML1* gene. Primers are indicated in blocks. Underlined are sequences of exons. Arrows indicate 5' to 3' direction of the primers (primers Ex_5F and Ex_5R). Complete sequences of the *AML1* gene (accession number D43968) was retrieved from GenBank database.

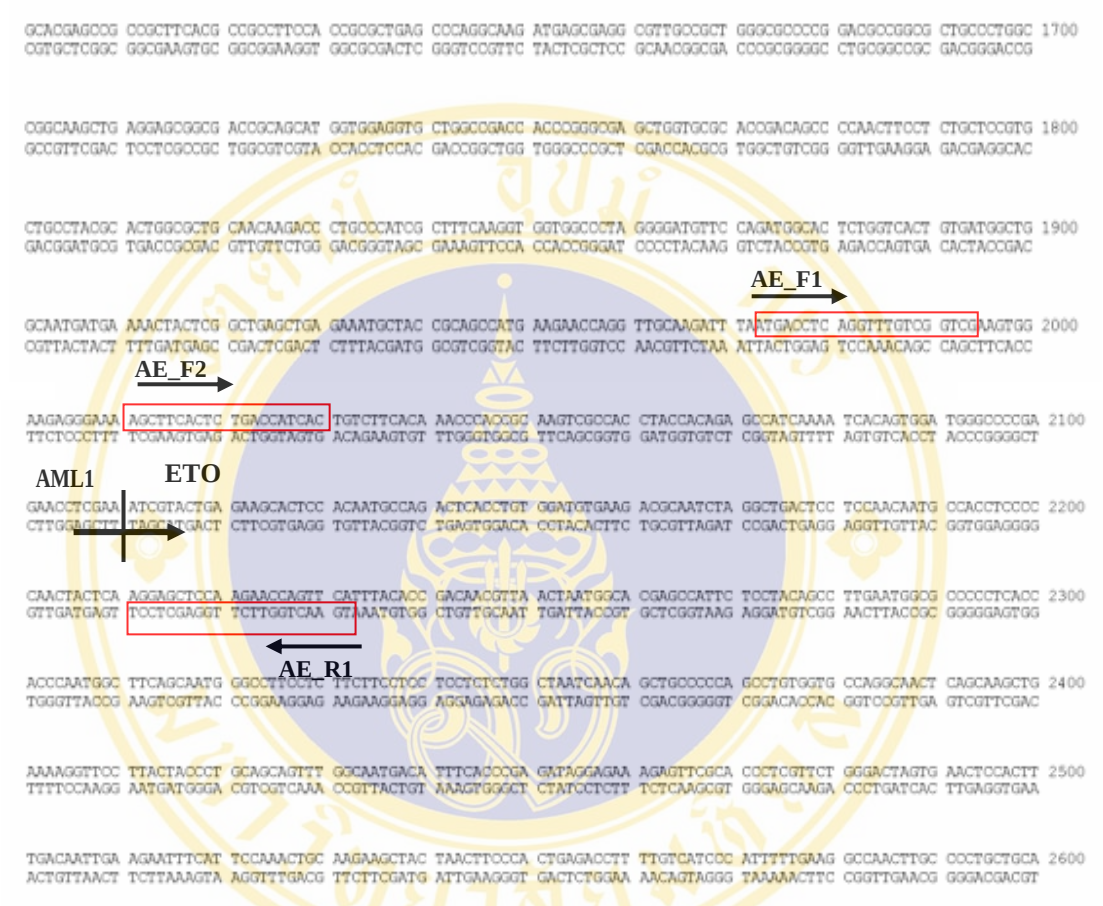


Figure 17 Nucleotide sequences of *AML1-ETO* fusion cDNA. The break point is indicated by a vertical line. Arrows indicate 5' to 3' direction of the primers (primers AE_F1, AE_F2 and AE_R1). The sequence data reported here have been deposited in GenBank sequence database under the accession number D13979.

Table 8 The nucleotide sequences of the primers for the amplification of *AML1* mutation and *AML1-ETO* fusion gene.

Fragment	Nucleotide sequence (5' - 3')	No. of nucleotide	Product size (bp)
1. *AML1* mutation			
1.1 Ex_3F	AGCTGTTTGCAGGGTCCTAA	20	336
1.2 Ex_3R	GTCCTCCCACCACCCTCT	18	
1.3 Ex_4F	CATTGCTATTCCTCTGCAACC	21	332
1.4 Ex_4R	CCATGAAACGTGTTTCAAGC	20	
1.5 Ex_5F	CCACCAACCTCATTCTGTTT	20	224
1.6 Ex_5R	AGACATGGTCCCTGAGTATA	20	
2. *AML1-ETO*			
2.1 AE_F1	ATGACCTCAGGTTTGTCGGTCG	22	261
2.2 AE_R1	TGAACTGGTTCTTGGAGCTCCT	22	223
2.3 AE_F2	AGCTTCACTCTGACCATCAC	20	
2.4 BA_F1	GTGGGGCGCCCCAGGCACCA	20	560
2.5 BA_R1	GTCCTTAATGTCACGCACGATTTC	24	

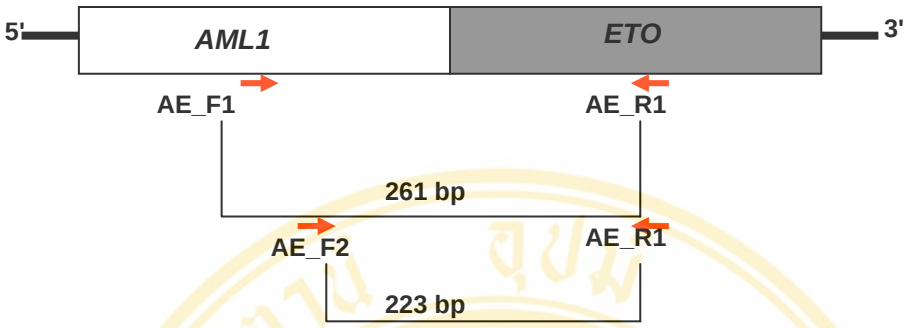


Figure 18 The schematic representation of the product of *AML1-ETO* fusion gene. Arrows indicate the location of the oligonucleotide primers and the direction of transcription.

2. Methods

2.1 Experimental strategy

BM or PB samples were diluted by adding an equal volume of phosphate buffered saline (PBS) at pH of 7.3 (see Appendix). Five to ten mL of the diluted samples were layered on Ficoll-hypaque reagent and then centrifuged at 2,000 rpm for 20 minutes in a swing-out rotor. The mononuclear cells (MNC) layer was collected and washed twice with PBS. The mononuclear pellets were stored in 1.5 mL microtube until use for nucleic acid preparation.

Total RNA and DNA were isolated from leukocytes by phenol/chloroform method. For the detection of *AML1-ETO* fusion, 108 AML patients were studied. The cDNA was synthesized by the reaction of AMV RT cDNA synthesis kit. The *AML1-ETO* fusion was detected by a semi-nested RT-PCR method using three primers. 414 AML patients were screened for *AML1* mutation by polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP). Abnormal SSCP bands were confirmed by an independent amplification and SSCP analysis. The sequencing analysis was performed to identify the nucleotide alteration and type of *AML1* mutation as well as to confirm the presence of *AML1-ETO* fusion and rule out the possibility of false positive results. All AML patients harboring *AML1* mutation or *AML1-ETO* were also studied for *FLT3* mutation. The results of *AML1* genotypes were correlated with immunophenotypes, karyotypes, and clinical data. The experimental strategy is shown in Figure 19.

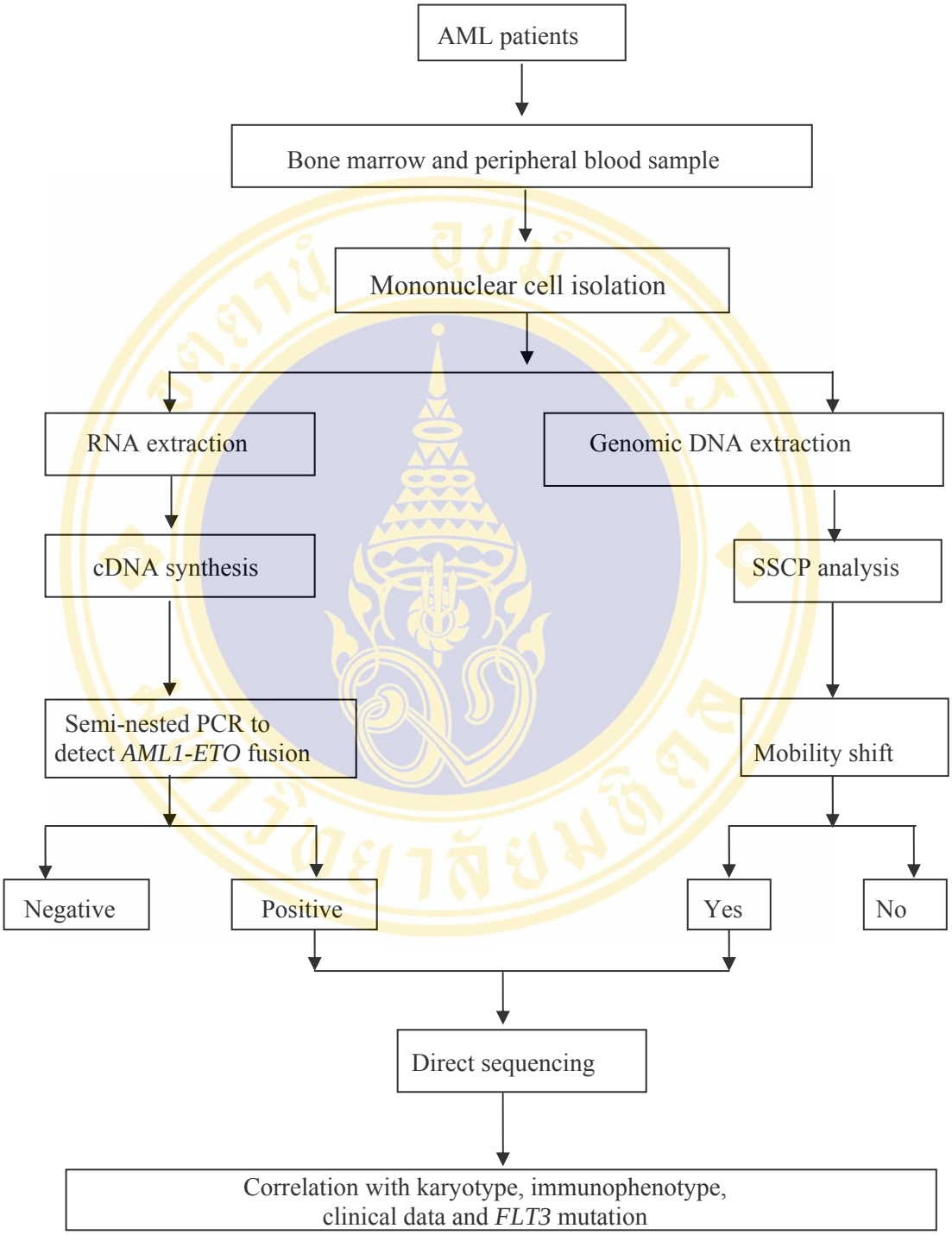


Figure 19 Flow chart of the experimental strategy used to study *AML1* abnormalities in this study

2.2 RNA preparation

Total RNA was extracted from MNC by a single-step method using TRIZOL™ reagent (Invitrogen corperation, CA, USA). One mL of TRIZOL™ reagent was added to lyse the MNC. The homogenized samples were incubated for 5 minutes at 15-30 ºC to permit the complete dissociation of nucleoprotein complexes. Two hundred microlitres of chloroform were added to 1 mL of TRIZOL™ regent, gently mixed for 15 seconds and incubated for 2-3 minutes, then centrifuged at 12,000xg for 15 minutes at 4 ºC. Following the centrifugation, the mixture was separated into a lower red phenol-chloroform phase, interphase, and a colorless upper aqueous phase. RNA, which was exclusively in the aqueous phase, was transferred to a fresh tube and the total RNA was precipitated by adding 0.5 mL of isopropyl alcohol per original 1 mL of TRIZOL™ reagent. Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000xg for 10 minutes at 4 ºC. The supernatant was discarded. The RNA pellet was then washed with 75% ethanol before centrifugation at 7,500xg for 5 minutes at 4 ºC. The RNA pellet was dried shortly. The RNA was dissolved in 20-50 µL in RNase-free water or sterile diethyl pyrocarbonate (DEPC)-treated water, depending on the amount of RNA. The concentration of the total RNA was estimated by using the following formula:

$$\text{Concentration of RNA (µg/ml)} = \frac{OD_{260} \times \text{dilution factor} \times 40}{1000}$$

The RNA was kept in DEPC-treated water at -20 ºC. For long-term storage, RNA was precipitated and kept in 75 % ethanol at -70 ºC.

2.3 Genomic DNA preparation

Genomic DNA was prepared from mononuclear cell (MNC) by standard phenol/chloroform method. After isolation of total RNA, the genomic DNA was harvested from the remaining suspension. Then precipitated by 300 µL of absolute ethanol. Then the DNA pellet was wash with 1 mL of 0.1 M of sodium citrate in 10% ethanol and centrifuged at 5,000xg for 5 minutes. Repeated this step twice times and wash with 1.5 mL of 75% ethanol for 10-20 minutes. DNA pellet was collected, air dried at room temperature and dissolved in an appropriate volume of sterile water.

The concentration of DNA was estimated from the measurement of OD_{260} and calculated by using following formula:

Concentration of DNA (ng/µl) = OD_{260} x dilution factor x 50

2.4 cDNA preparation

cDNA was synthesized by the reaction of AMV RT cDNA synthesis kit (Promega Corporation, USA). The reverse transcription reaction was primed with either oligo $(dT)_1$ or random primers. One µg of mRNA was placed in a mixture reaction that contained 5 mM $MgCl_2$, 1x reverse transcription buffer, 1 mM each dNTP, 1 u/µl recombinant RNasin® ribonuclease inhibitor, 15 u/µl AMV reverse transcriptase, 0.5 µg oligo $(dT)_{15}$ or random primers per 1 microgram of mRNA. The cDNA was synthesized at 42 ºC for 45 minutes. The first-strand cDNA was kept in -20 ºC.

2.5 Detection of *AML1-ETO* fusion by semi-nested RT-PCR

The *AML1-ETO* fusion gene was detected by the semi-nested RT-PCR method. Two pairs of primers were designed. The first pair was used for the first round PCR and the second pair was used for the second round PCR (nested PCR) to enhance the sensitivity of the assay. The integrity of RNA preparation was confirmed by RT-PCR amplification of β–actin mRNA in the same reaction of AML patients.

First round PCR reaction mixture was made up in the total volume of 25 µL with the following constituents, 0.5 U Taq DNA polymerase, 1x Taq polymerase buffer, 1.75 mM $MgCl_2$, 0.4 µM of AE_F1 and AE_R1 oligonucleotide primers, 0.625 µM of BA_F1 and BA_R1, and 4 mM dNTP. cDNA was then synthesized from 1 µg of total RNA. The reaction mixture was briefly centrifuged and then placed in a thermal cycler with the PCR profile as follows: initial denaturation at 94 °C for 5 minutes and 35 cycles of denaturation at 93 °C for 1 minute, annealing at 63 °C for 1 minute and extension at 72 °C for 1 minute with post extension step at 72 °C for 5 minutes. PCR products were visualized on ethidium bromide-stained 2% agarose gels. A second round, semi-nested PCR was performed on 1 µL of the first round PCR

product for 35 cycles under the same condition as the first round PCR but with 0.4 μM of AE_R1 and AE_F2 oligonucleotide primers. Water and normal cDNA were used as controls for PCR steps. AML patients with *AML1-ETO* were used as a positive control.

2.6 Screening of *AML1* mutation by PCR-SSCP analysis

PCR-SSCP technique was used to screen for *AML1* mutation in the exons 3, 4 and 5 which correspond to the runt domain. PCR was performed with 100 ng of genomic DNA, 1x PCR buffer, 1.5 mmol/L $MgCl_2$, 10 μmol/L dNTP, 10 pmol of each primer, 0.5 U of Taq DNA polymerase (Immolase, Bioline, Germany) in a total reaction volume of 25 μL. PCR amplification was performed on a Thermal Cycler (Perkin Elmer PCR2400, USA) under the following conditions: initial denaturation at 94 °C for 5 minutes, 35 cycles at 94 °C for 30 seconds, 62°C for 1 min (exon 3) or 57 °C for 1 min (exon 4 and 5) and 72 °C for 1 min, followed by a final elongation at 72 °C for 5 min. For the SSCP analysis, 2 μL of the PCR product was mixed with 8 μL of formamide dye. The products were heated at 95 °C for 10 minutes then cooled on ice for 10 minutes, then applied to 10% nondenaturing polyacrylamide containing 5% (v/v) glycerol. The 10% polyacrylamide gel was prepared by mixing 2.5 mL of stock 40% acrylamide-bisacrylamide (49:1), 2 mL of 5x Tris-borate-EDTA (TBE) buffer, 500 μL of glycerol, 4.93 mL of distilled water, 70 μL of 10% ammonium persulphate, and 4 μL of tetramethyl etylenediamine (TEMED) in a total volume of 10 mL. Electrophoresis was carried out at 150 V for 3 hours (exons 3 and 5) or 200 V for 2 hours (exon 4) in 1x TBE buffer (Table 9). The silver staining method was performed to visualize SSCP bands. To control for SSCP conditions, DNA from normal controls was also studied in the same condition as leukemic samples. All PCR products with abnormal SSCP bands were confirmed by an independent amplification and SSCP analysis.

2.7 Silver staining

The gel was fixed in 50 mL of 40% methanol for 10 minutes and 160 mM HNO_3 for 6 minutes, then rinsed and washed twice in deionized water for 5 minutes.

After that, 50 mL of the silver staining solution (0.2% W/V $AgNO_3$) were added and the gel was left to stain for 20 minutes each time. Fifty milliliters of developer (3% W/V Na_2CO_3 and formaldehyde) were added into the gel and shaking for 5 minutes. The solution was discarded and the gel was rinsed and washed with deionized water. The gel was also dried on a sheet of filter paper in a slab gel-dryer at room temperature for 24-48 hours.

Table 9 The PCR-SSCP conditions for the detection of *AML1* mutation.

Fragment	Product size (bp)	PCR condition	SSCP condition		
		Annealing temp (ºC) and additive solution	Polyacrylamide-gel (%)	Duration of eletrophoresis (hours)	Temp (ºC)
Exon 3	336	62 + 10% DMSO	10	3	RT
Exon 4	332	57	10	2	RT
Exon 5	224	57	10	3	RT

DMSO: Dimethyl sulfoxide

RT: Room temperature

2.8 Sequencing of PCR products

2.8.1 Purification of PCR products

All PCR products with abnormal SSCP bands or *AML1-ETO* positive bands were purified and sequenced in both directions to identify the type of mutations. The aberrant products were separated by electrophoresis on 2% agarose gel and cut with a sharp blade. The selected bands were placed into a 15-mL centrifuge tube. The volume of gel was estimated from its weight and three volumes of QG buffer (QIAGEN, Chatsworth, CA, USA) were added to the gel. The gel was then incubated at 50 °C for 10 minutes in a waterbath until gel completely dissolved. One gel volume of isopropanol was added. After that, the mixture was applied to QIAquick column and the column was then placed in a 1.5 mL collection tube. The column set was spun at 12,000xg for 1 minute whereby the PCR products were trapped by the column. The residual agarose was removed from the column by adding 0.5 mL of QG buffer and centrifuged again. The column was washed with 0.75 mL of PE buffer and spun again. The PCR products were eluted from the column by soaking with 10-30 µL of elution buffer or distilled water for 1 minute and the products solution was collected into a 1.5 mL microtube by centrifugation at 12,000xg for 1 minute. The purified product was run on agarose gel electrophoresis and its concentration was estimated from band intensity by comparing to standard DNA markers, Φx174 DNA digested with HaeIII.

2.8.2 Cycle sequencing

The nucleotide sequences of the purified PCR product were analyzed by the automated sequencer, MegaBACE^{TM} 1000 Automated DNA Sequencer (Amersham Life Science, USA). The cycle sequencing reaction was performed using the DYEnamic^{TM} ET Dye Terminator Cycle Sequencing Kit for MegaBACE^{TM} (Amersham Pharmacia Biotech, UK) following the manufacturer's protocol.

The reaction mixture was made by mixing 4 µL terminator ready reaction mix, 3.2 pmol primer, 30-40 ng of purified PCR product (as DNA template), and distilled water to a total volume of 20 µL. Using a Thermal Cycler (Perkin Elmer PCR 2400, USA), the DNA template in a mixture was amplified for 25 cycles, each of which consisted of 96 °C for 10 second, 50 °C for 5 second, and 60 °C for 4 minutes.

2.8.3 Preparation of cycle sequencing product for loading

The cycle sequencing product was purified from the excessive dye terminators by precipitation with 2 µL of 3 M sodium acetate, pH 4.6, and 50 µL of absolute ethanol. The pellet was rinsed with 250 µL of 70% ethanol and dried in the dark place for 10-15 minutes. The suspension was then loaded to the MegaBACE^{TM} 1000 Automated DNA Sequencer (Amersham Life Science, USA).

2.8.4 Analysis of DNA sequence

The sequencing product suspension was automatically injected into the capillary for 30 seconds at 2.5 kV and subjected to capillary electrophoresis in the POP-6 polymer for 2 hours at 12.2 kV, 50 °C. Raw electrophoregram was collected using the MegaBACE Sequence Analyzer 3.0.0111.1603 (Amersham Bioscience, USA). The sequence data was examined by Sequencer Navigator and analyzed by MacVector 4.5.3 software.

2.9 *FLT3* mutation analysis

FLT3 analysis was investigated in 16 AML patients with *AML1-ETO* fusion and 16 AML patients with *AML1* point mutation. The methods of *FLT3* mutation analysis were performed according to the methods previously established (101).

2.9.1 Polymerase chain reaction analysis of *FLT3* internal tandem duplication (ITD) mutation

FLT3-ITD was determined by the amplification of exon 14 to exon 15. PCR reaction was performed using a 25 µL PCR mixture which contained 50 ng of DNA template, 2.5 mL of 10x PCR buffer, 0.5 µL of 50 mM of $MgCl_2$, 0.5 µL of 10 mM of dNTP, 1 µL of 10 pmol/µl of each 11F and 12R primers, 0.125 of 1U/µL of *Taq* DNA polymerase, and 17.375 µL of sterile distilled water.

PCR was performed in the Thermal Cycler under the following conditions: i) initial denaturation at 94 °C for 12 minute, ii) 30 cycles of denaturation at 94 °C for 1 minute, iii) annealing at 59 °C for 1 minute and extension at 72 ° C for 2 minute, and iv) final extension at 72 °C for 10 minutes. The PCR products were visualized by electrophoresed with 2-2.5 % agarose gel at 100 volts for approximately

60 minutes. The gel was stained in a 2 µg/mL ethidium bromide solution and the PCR products were viewed on gel documentation machine.

2.9.2 PCR-RFLP analysis for the detection of *FLT3* tyrosine kinase domain (TKD) mutation

Exon 20 of *FLT3*, a part of tyrosine kinase domain, was amplified to detect FLT3-TKD mutation. PCR reaction contained 100 ng of DNA template, 2.5 µL of 10x PCR buffer, 0.5 µL of 50 mM of $MgCl_2$, 0.5 µL of 10 mM of dNTP, 1 µL of 10 pmol/ul each of 17F and 17R primers, 0.125 of 1U/uL of *Taq* DNA polymerase, and 17.375 µL of sterile distilled water. PCR reactions were performed for 30 cycles in the Thermal Cycler. The details of PCR cycles were as followed (i) denaturation at 94 °C for 30 seconds (7 minutes for the first cycle), (ii) annealing at 59 °C for 30 seconds, (iii) extension at 72 ° C for 1 minute (5 minutes for the last cycle). Electrophoresis was performed in 2% agarose gel to check the PCR product.

The PCR products were digested with restriction enzyme *EcoR*V. The digestion reaction was carried out in 20 µL which contained 10-15 µL of PCR products, 1 µL of 5x *EcoR*V buffer, 0.25 µl of 1U/µL *EcoR*V enzyme followed by adding sterile distilled water to final volume of 20 uL. The mixture was incubated at 37°C overnight. After complete digestion, the digested products were fractionated through 2.5% agarose gel electrophoresis.

2.10 Flow cytometric analysis of leukemic samples

Mononuclear cells (MNC) were prepared from bone marrow or peripheral blood by using Ficoll-hypaque density gradient centrifugation. MNCs were stained with various monoclonal antibodies (mAbs) labled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP). These labeled monoclonal antibodies against the following antigens.: CD2, CD3, CD5, CD7, CD10, CD11c, CD13, CD14, CD16, CD19, CD20, CD22, CD33, CD34, CD41a, CD45, CD56, CD117, glycophorin A (GlyA), anti MPO and TdT (Table 7). Cytoplasmic antigens (MPO and TdT) were stained after permeabilizing the cell with FACS™ Permeabilizing solution (Becton Dickinson, San Jose, CA, USA). Analysis was

performed using FACScalibur flow cytometry (Becton Dickinson). An antigen was considered positive when the gated cells expressed at least 20% of antigen (102).

2.11 Chromosome analysis of leukemic samples

Chromosome studies were performed at the DNA Center, Bangkok. Conventional chromosome banding studies were performed using standard techniques and chromosomal abnormalities were described according to the International System for Cytogenetic Nomenclature (ISCN) (103).

2.12 Statistical analysis

The Mann-Whitney test was used to compare quantitative variable. Comparison of qualitative variables was performed using a Fisher's exact test (74). For all analyses, a p-value of less than 0.05 was considered statistically significant.

CHAPTER V
RESULTS

1. Analysis of *AML1-ETO* fusion gene in Thai adult AML patients

One hundred and eight consecutive samples of AML patients diagnosed in the year 2004-2005 were available for RT-PCR analysis of *AML1-ETO* fusion gene. They were classified as follow: 2 cases of M0, 18 cases of M1, 36 cases of M2, 23 cases of M3, 18 cases of M4, 8 cases of M5, and 3 cases of M6

1.1 *AML1-ETO* fusion gene detected by RT-PCR

RT-PCR analysis for the detection of *AML1-ETO* fusion in 108 Thai AML patients was performed. The specificity of the PCR products was confirmed by sequencing the semi-nested products across the molecular breakpoint. The fusion gene contained the 5'*AML1* gene fused with the 3' part of the *ETO* gene. The integrity and quality of synthesized cDNA was assessed by RT-PCR amplification of the β-actin gene in the same reaction of *AML1-ETO* fusion gene. Figure 20 shows the PCR products of 16 AML patients who were found to carry *AML1-ETO* fusion gene. The length of *AML1-ETO* and β-actin amplified product were 223 bp and 560 bp, respectively.

Figure 21 shows the nucleotide sequences of *AML1-ETO*. The breakpoints of *AML1-ETO* fusion gene located at exon 5 of *AML1* gene and exon 1 of *ETO* gene which generated a fusion product of 223 bp. RNA of normal healthy controls and positive controls were also reverse-transcribed and amplified in parallel with all leukemia samples.

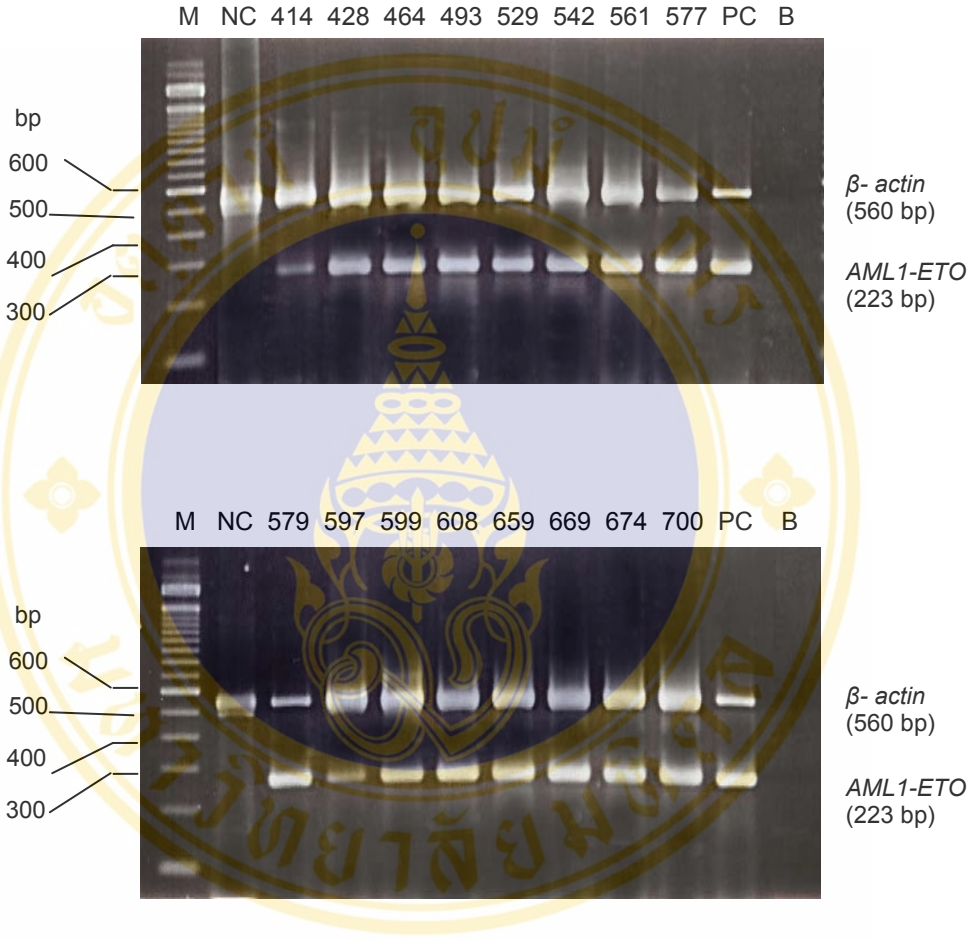


Figure 20 Agarose gel electrophoresis of 16 AML patients with *AML1-ETO* fusion gene. Lanes PC, NC and B are positive control, negative control and blank, respectively. Lane M is a 100-bp DNA ladder used as a standard-size marker.

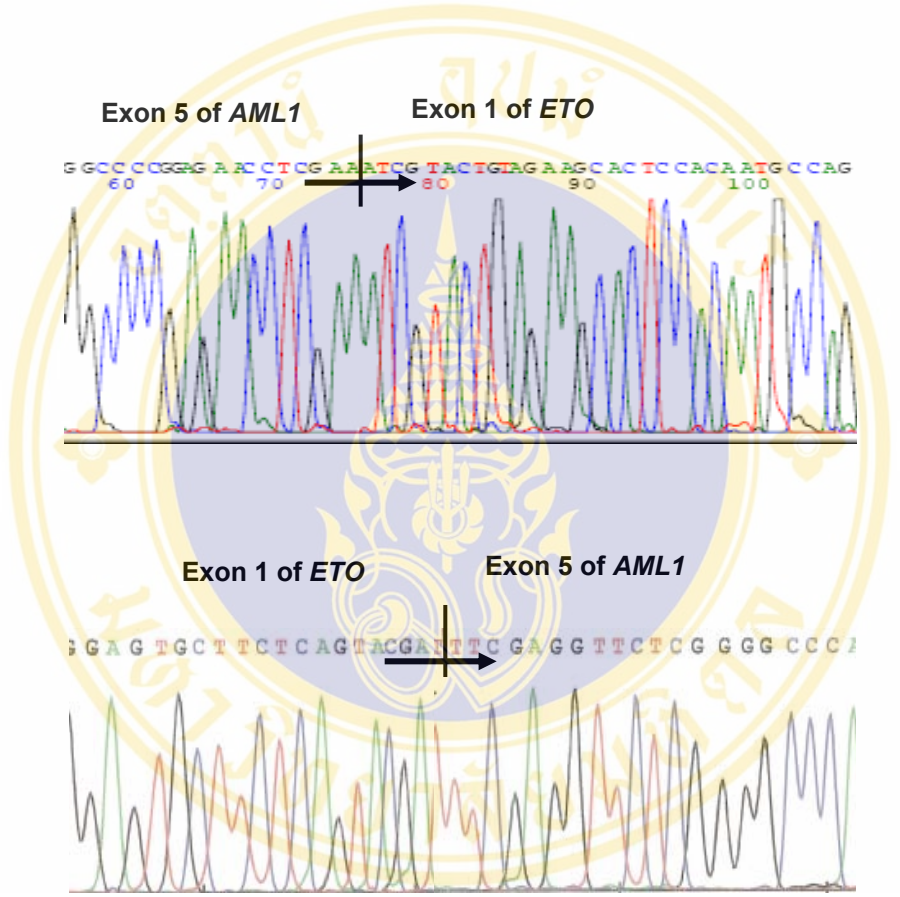


Figure 21 Sequencing analysis of *AML1-ETO* fusion gene. (A) Nucleotide sequences of a positive control, sequenced by a forward primer (5'-3') and (B) Patient no. 561 sequenced by a reverse primer (5'-3').

1.2 The frequency of *AML1*-ETO fusion gene in Thai adult AML patients

The *AML1-ETO* was detected in 16 out of 108 AML patients (15%) as shown in Table 10. According to the FAB Classification, most of the *AML1-ETO* fusion gene was detected in AML-M2 subtype (38%). One patient each in M4 (6%) or M5 subtype (13%) subtype had the fusion gene while no cases of M0, M1, M3, and M6 AML did. Fifteen patients with *AML1-ETO* detected by RT-PCR were found to have t(8;21)(q22;q22) by karyotypic analysis. One patient had a normal karyotype (no.561).

Table 10 The frequency of *AML1-ETO* in Thai AML patients (n=108)

FAB subtype	No. studied	Presence of AML1-ETO	% of total cases studied
M0	2	0	0
M1	18	0	0
M2	37	14	38
M3	22	0	0
M4	18	1	5.5
M5	8	1	12.5
M6	3	0	0
Total	108	16	15

1.3 Clinical characteristics of AML patients with *AML1-ETO* fusion gene

Table 11 shows the characteristics of AML-M2 patients with *AML1-ETO* (*AML1-ETO* positive) compared with AML-M2 patients who did not find *AML1-ETO* (*AML1-ETO* negative). The majority of patients with *AML1-ETO* were young to middle-aged adults with the median age of 36 years (range 15-59). Eleven males and 5 females (male to female ratio of 2.2) were found in this cohort. Interestingly, the patients with *AML1-ETO* showed a significantly lower median age than those without *AML1-ETO* (p=0.006). Forty three percent of patients with *AML1-ETO* were younger than 30 years old. The median white blood cell (WBC) count in the *AML1-ETO* positive group was relatively not highly elevated (median, $20x10^9$/L) and the majority of cases had the WBC count below $50x10^9$/L, except for three patients. Eleven of 16 patients with *AML1-ETO* (69%) were moderately to markedly pale and the hematocrits (Hct) were mostly below 25%. The platelet (Plt) counts were slightly lower in the *AML1-ETO* positive group with the median of $28x10^9$/L. The median blast counts were not different between the two groups, with or without *AML1-ETO*.

Table 11 Clinical features of AML-M2 patients with or without *AML1-ETO* fusion gene.

Parameter	***AML1-ETO*** **positive (n=16)**	***AML1-ETO*** **negative[1](n=22)**	**P value**
Median age (years)	36.0	43.00	0.006[2]
Median Hb (g/dL)	8.20	8.10	0.485
Median Plt ($x10^9$/L)	28.00	40.00	0.189
Median Hct (%)	23.60	24.70	0.727
Median WBC ($x10^9$/L)	20.30	26.00	0.366
Median RBC ($x10^9$/L)	3.08	2.79	0.799
Median % blast	55.00	52.00	0.666

[1]Patients with the following karyotypes, 14 normal karyotype, 1 del 5, 1 del 9, and 6 patients with failed karyotypic analysis.

[2]Statistical significance

1.4 Association of *AML1-ETO* fusion gene with karyotypes

All AML patients who had t(8;21)(q22;q22) were found to have the *AML1-ETO* fusion gene as detected by RT-PCR analysis. Additionally, *AML1-ETO* was also detected in one patient with a normal karyotype. Interestingly, 7 out of 16 cases (41%) with t(8;21) and *AML1-ETO* had loss of a sex chromosome (X loss in 2 females and Y loss in 5 males). Two patients with t(8;21) and loss of Y chromosome also had t(4;15)(p14;q13) or the addition of chromosomal band 17(p13). One AML-M2 with *AML1-ETO* had also a deletion of the long arm of chromosome 9(q22q33).

1.5 Association of *AML1-ETO* with immunophenotypes

Immunophenotypic findings of 14 AML-M2 patients with *AML1-ETO* fusion gene were compared to 22 AML-M2 with a normal karyotype or other molecular abnormalities. Table 12 shows the antigenic expression of AML-M2 patients with and without *AML1-ETO*. High proportion of patients with *AML1-ETO* expressed myeloid antigens including CD11c (85.7%), CD13 (100%), CD33 (93%), CD34 (78.4%), CD64 (54.5%), and CD117 (100%). Additional antigens that were highly expressed in AML patients with *AML1-ETO* were HLA-DR (93%) and MPO (93%). Patients with *AML1-ETO* had a higher incidence of CD56 and CD117 expression ($p<0.05$) as compared to those without *AML1-ETO*. Patients with *AML1-ETO* had a higher intensity of CD56 (median expression, 61% vs 12%) ($p=0.034$) but slightly lower CD33 (median expression, 86.4% vs 96.5%) ($p=0.02$) as compared to patients lacking *AML1-ETO* (Figure 22).

Figures 23 and 24 show the flow cytometric analysis and morphological features of a representative AML-M2 patient who had t(8;21) with *AML1-ETO* (no. 597) and AML-M2 with a normal karyotype but no detectable *AML1-ETO* (no.474), respectively.

Table 12 Immunophenotypic analysis of AML-M2 patients with (n=14) or without *AML1-ETO* fusion (n=22)

Antigen	AML1-ETO positive (n=14)			AML1-ETO negative (n=22)			P value (median expression)	P value (% positive)
	Positive cases / total cases studied	% positive of total cases	Median expression	Positive cases / total cases studied	% positive of total cases	Median expression		
CD2	0/6	0	0.20	0/10	0	0.49	0.3	1.0
CD3	0/14	0	2.50	0/10	0	2.40	0.413	1.0
CD5	0/14	0	0.29	1/13	7.7	0.72	0.096	0.367
CD7	1/14	7.1	0.59	3/14	21.4	1.02	0.345	0.628
CD10	0/12	0	0.15	0/12	0	0.3	0. 450	0.158
CD11c	13/14	93	39.98	20/22	91	63.15	0.130	1.0
CD13	14/14	100	62.22	21/22	95	87.52	0.558	1.0
CD14	0/14	0	1.88	0/22	0	2.06	0.562	0.960
CD16	0/14	0	5.65	1/17	5.9	2.90	0.440	0.538
CD19	7/14	50	17.53	9/22	40.9	19.87	0.689	0.309
CD20	0/13	0	0.27	0/21	0	0.39	0.300	1.0
CD22	0/13	0	1.69	3/22	13.6	4.13	0.457	0.06
CD33	13/14	93	74.42	19/20	95	96.54	0.02*	1.0
CD34	11/14	78.6	43.70	8/22	36.4	19.81	0.344	0.257
CD41a	0/14	0	0.56	0/22	0	2.15	0.400	1.0
CD56	12/14	85.7	61.15	10/21	47.6	12.21	0.034*	0.03*
CD64	6/11	54.5	38.68	11/17	64.7	42.25	0.692	0.749
CD117	12/12	100	53.98	11/20	55	39.13	0.454	0.05*
HLA-DR	13/14	93	72.7	17/22	77.3	52.82	0.496	0.408
GPA	0/12	0	0.58	0/22	0	1.49	0.450	0.960
MPO	13/14	93	73.11	19/21	90.5	31.6	0.10	0.534
TdT	0/14	0	0.86	1/22	4.5	0.48	0.148	0.675

* Statistical significance

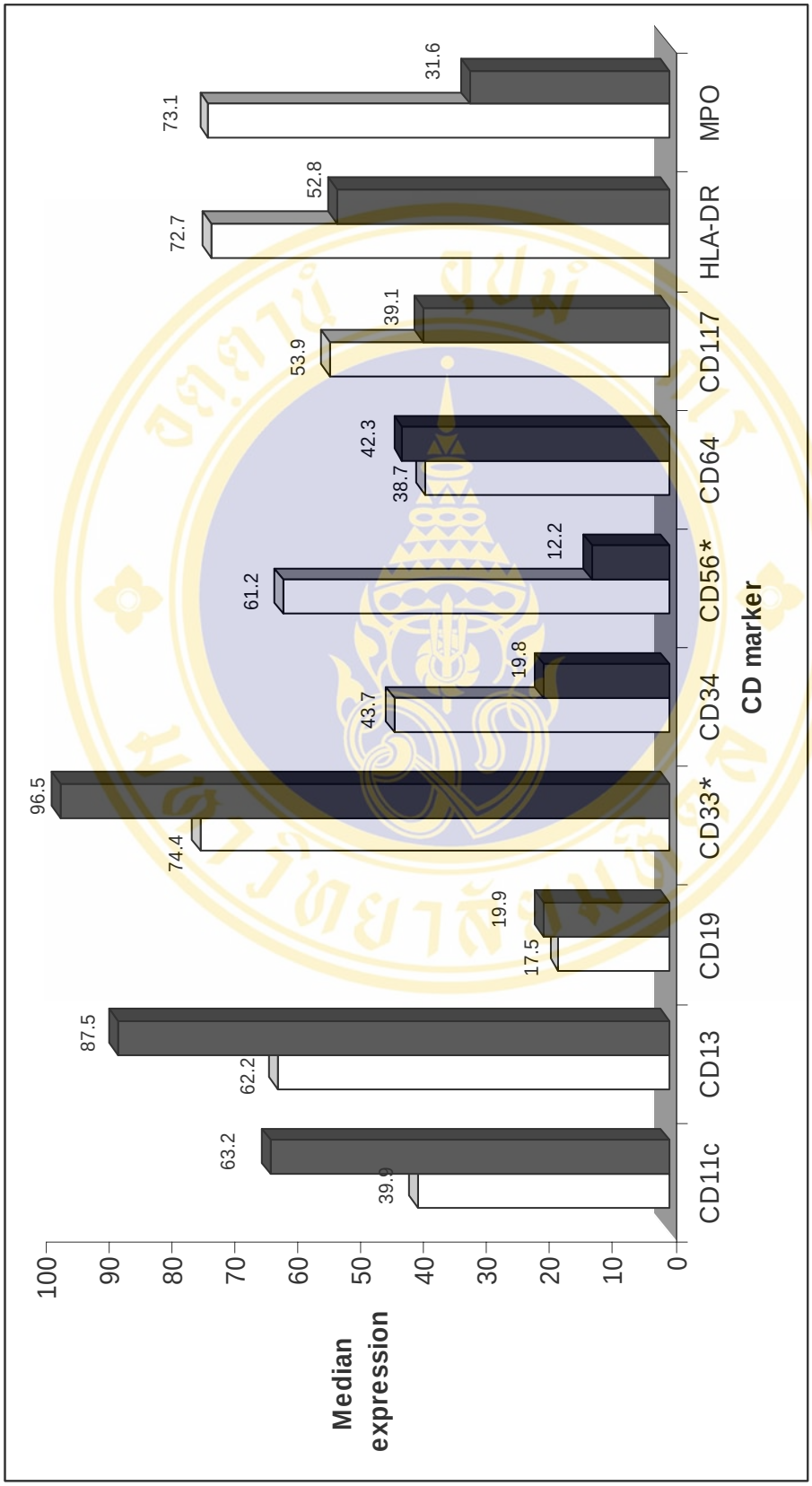


Figure 22 Comparison of the median expressions of CD markers between AML-M2 patients with ***AML1-ETO*** (black bar) and those without ***AML1-ETO*** (white bar) *****Statistical significance ($p<0.05$)

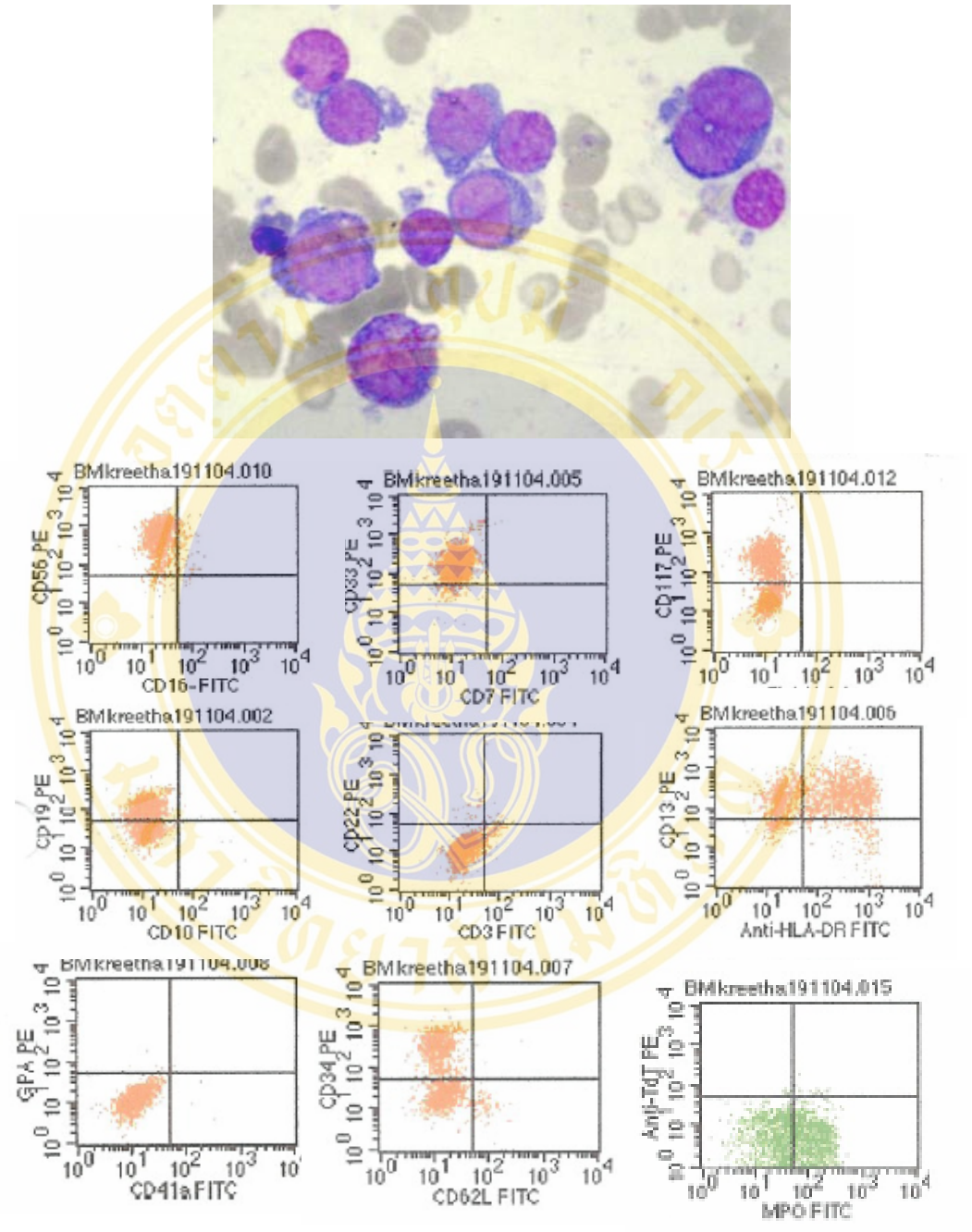


Figure 23 Immunophenotypic markers and morphological features of a representative AML-M2 patient with *AML1-ETO* by RT-PCR and t(8;21) by karyotyping (no. 414).

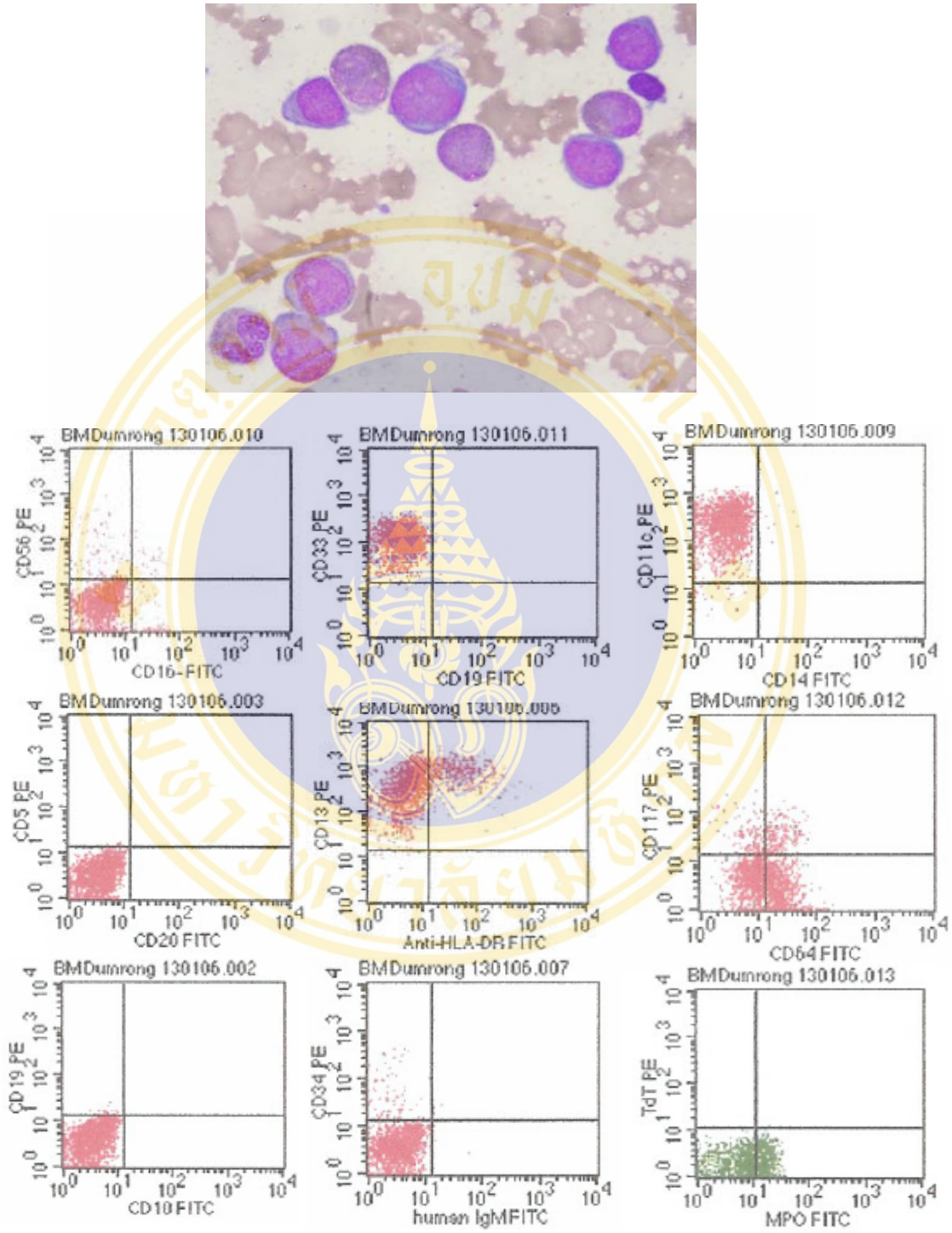


Figure 24 Immunophenotypic markers and morphological features of a representative AML-M2 without *AML1-ETO*, who had a normal karyotype.

1.6 Association studies of *AML1-ETO* fusion gene and *FLT3* mutation

Sixteen patients with *AML1-ETO* were studied for *FLT3* mutations in both TKD and ITD mutations. Only one patient who had *AML1-ETO* was found to have a TKD mutation. The amplified product of the wild-type was digested into 2 bands (68 bp and 46 bp) by *EcoR*V whereas the amplified products containing TKD mutation had an undigested band of 114 bp (no. 529) as shown in Figure 25. No patients with *AML1-ETO* were found to have an ITD mutation. The summary of all 16 AML patients with *AML1-ETO* and associated karyotypes and *FLT3* genotype was delineated in Table 13.

Figure 25 Agarose gel electrophoresis of AML patients with *AML1-ETO* who had also found a *FLT3* TKD mutation (no.529). The amplified products of the wild-type were digested into 2 bands (68 bp and 46 bp) by *EcoR*V whereas the amplified products containing TKD mutation had an undigested band (114 bp). Lane M is a 100-bp DNA ladder used as a standard-size marker.

Table 13 Main features of 16 AML patients with *AML1-ETO* fusion

No.	Subtype	Sex	Age	Hb	Hct	WBC	Plt	Karyotype	FLT3 genotype
414	AML-M2	M	15	3.80	10.80	36.10	31.00	45,X, t(8;21)(q22;q22)	No ITD & TKD
428	AML-M2	M	38	10.30	30.10	57.00	15.80	45,X, t(8;21)(q22;q22)	No ITD & TKD
464	AML-M2	F	26	11.00	31.00	15.70	46.30	45,X, t(8;21)(q22;q22)[9].	No ITD & TKD
493	AML-M2	M	43	9.20	27.20	8.30	54.00	46,XY, t(8;21)(q22;q22),del(9)(q22q33)	No ITD & TKD
529	AML-M2	F	59	6.7	21.8	21.1	14.00	46,XX, t(8;21)(q22;q22)	TKD
542	AML-M2	M	52	14.20	30.00	92.78	67.00	45,X, t(8;21)(q22;q22),add(17)(p13)	No ITD & TKD
561	AML-M2	M	16	8.20	23.60	3.03	117.00	46,XY, Normal male karyotype.	No ITD & TKD
577	AML-M2	M	29	8.50	25.00	18.30	35.00	45,X, t(8;21)(q22;q22),t(4;15)(p14;q13)	No ITD & TKD
579	AML-M2	F	39	3.70	18.50	4.00	140.00	46,XY, t(8;21)(q22;q22).	No ITD & TKD
597	AML-M2	M	17	9.20	26.90	2.91	28.00	46,XY, t(8;21)(q22;q22)	No ITD & TKD
599	AML-M2	M	40	13.00	38.00	28.00	20.00	46,XY, t(8;21)(q22;q22)	No ITD & TKD
608	AML-M2	M	30	11.00	35.10	21.46	21.00	45,X, t(8;21)(q22;q22)	No ITD & TKD
659	AML-M4	F	44	6.50	20.00	101.00	12.00	46,XX, t(8;21)(qq22;q22)	No ITD & TKD
669	AML-M5	M	55	7.50	22.40	20.30	5.00	46,XY, t(8;21)(q22;q22).	No ITD & TKD
674	AML-M2	M	19	6.10	17.40	14.90	20.00	46,X, t(8;21)(q22;q22)	No ITD & TKD
700	AML-M2	F	46	7.50	22.90	27.00	2.00	45,X, t(8;21)(q22;q22)	No ITD & TKD

2. Analysis of *AML1* mutation in 414 Thai adult AML patients

2.1 FAB classification of 414 AML patients

According to the FAB criteria, 414 Thai adult *de novo* AML cases could be subsequently classified into 8 subtypes. As shown in Table 14, the major subtypes were M1 (23.7%), M2 (27.5%), M3 (18.9%), and M4 (16.2%). M0, M5, M6, and M7 were infrequently found accounted for 1.7%, 7.7%, 1.9%, and 0.2%, of all cases, respectively.

Table 14 FAB classification of 414 Thai adult *de novo* AML patients.

AML subtype	No. studied	% of total cases
M0	7	1.7
M1	98	23.7
M2	114	27.5
M3	78	18.9
M4	67	16.2
M5	32	7.7
M6	8	1.9
M7	1	0.2

2.2 Cytogenetic profile of 414 AML patients

Of 414 AML cases, about half had a normal karyotype as shown in Table 15. According to the WHO criteria, AML with recurrent translocations were accounted for 17.1% of all cases, including 9.4% of t(8;21), 4.1% of t(15;17), 2.2% of inv(16), and 1.2% of chromosome 11q23 abnormalities. The t(16;21), monosomy 7, deletion of chromosome 9, trisomy 8 and trisomy 21 or 21q+ were found in 0.7%, 3.9%, 2.2%, 3.1%, and 1.7%, respectively. The t(9;22) or monosomy 5 was each found in 2% of all cases.

Table 15 Incidence of chromosome abnormalities in 414 Thai Adult AML patients[1].

Karyotype	No. of studied	% of total cases
t(8;21)	39	9.4
t(15;17)	18	4.3
inv(16)	9	2.2
11q23 abnormalities	5	1.2
t(9;22)	8	2.0
t(16;21)	3	0.7
-5	8	2.0
-7	16	3.9
del(9)	9	2.2
+8	13	3.1
+21/21q+	7	1.7
Normal karyotype	195	47.1
Other abnormalities	44	10.7
Chromosome analysis not performed	41	9.9

[1]Performed by Cytogenetic Laboratory, DNA Center, Bangkok

2.3 *AML1* mutation detected by PCR analysis

Exons 3, 4 and 5 of *AML1* gene, responsible for the runt domain of AML1 protein were amplified using primers designed to be specific for each fragments. After amplification, an aliquot of PCR product was examined by agarose gel electrophoresis. The size of exons 3, 4 and 5 was 336, 332 and 224 bp, respectively. Figure 26 shows the PCR products of gene obtained from 16 AML patients who had *AML1* mutation, including six each involving exon 3 or exon 4 and one of exon 5. Additionally, two patients had *AML1* mutations in intron 5 and one in intron 3. Sample no. 16 and 159 (exon 5) had two different sizes of PCR fragments. The larger fragment was wild-type and the smaller fragment was mutant with deleted 17 nucleotide bases. Sample no. 105, 219, and 342 (exon 4) showed one extra mutant fragment. Interestingly, one heteroduplex formation between the wild type and the mutant *AML1* could be seen in case no. 460. All extra bands were contained nucleotide insertions. Nine nucleotide bases were inserted in DNA fragment obtained from patient no. 105 and 219. DNA obtained from patient no. 342 was inserted by 5 nucleotide bases while no. 460 had a duplicated 22 nucleotide bases in the DNA fragment of the exon 4.

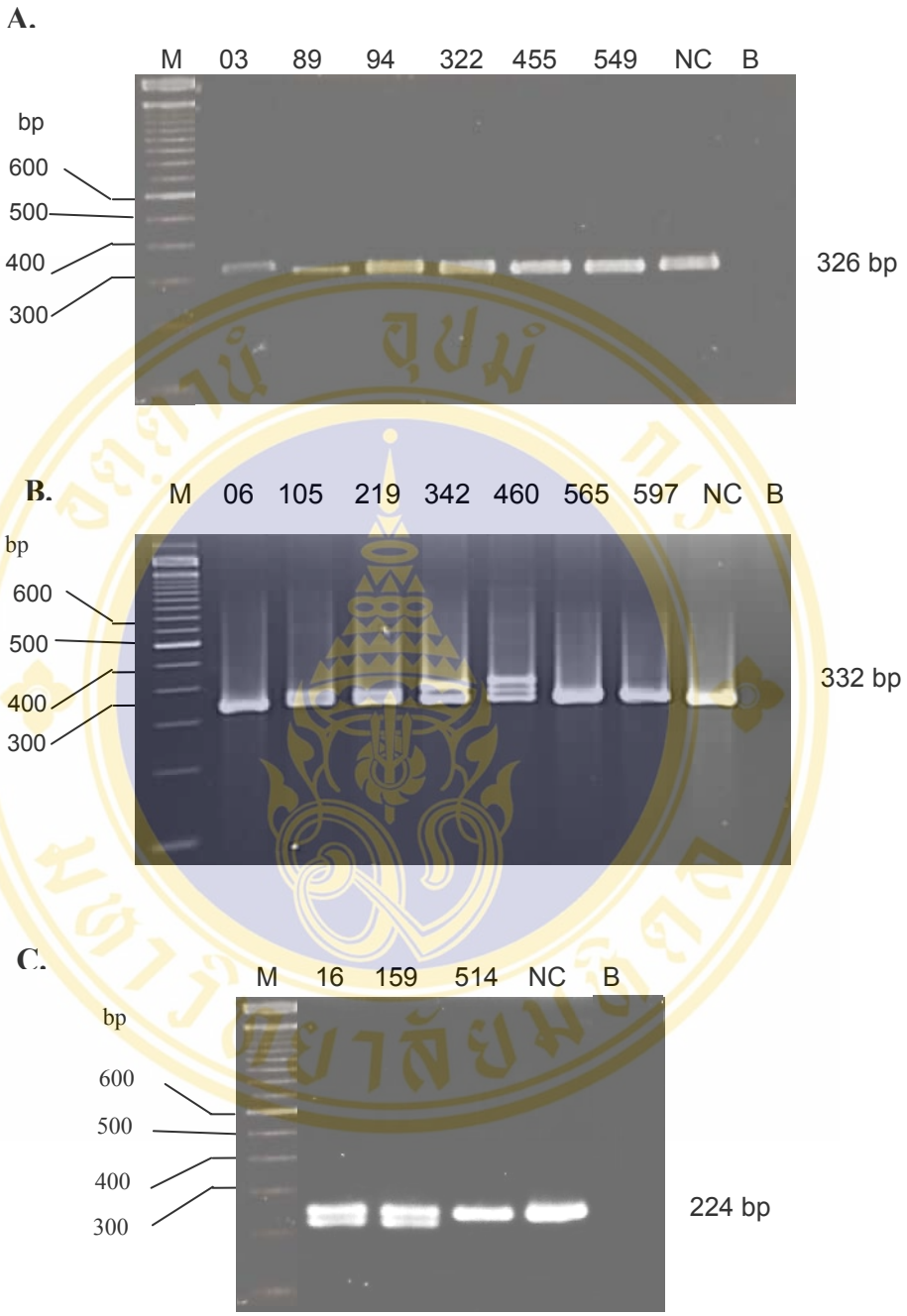


Figure 26 Agarose gel electrophoresis of PCR products from 16 cases with *AML1* mutation. Panels A, B and C represent the PCR products of 16 patients with *AML1* mutation in exons 3, 4, and 5, respectively. Lane M is a 100-bp DNA ladder used as a standard-size marker. Lanes NC and B represent a normal and blank control, respectively.

2.4 *AML1* mutation detected by SSCP and sequencing analysis

In order to screen for *AML1* mutation, SSCP was performed in 414 cases. All mobility patterns of AML patients were compared with normal controls. Figure 27 shows SSCP analysis and sequencing analysis of six patients with *AML1* mutation in exon 3, including one duplication (no. 003), one deletion (no. 549), and 4 cases of C to T base substitution (no. 089, 094, 322, and 455). One duplication within intron 3 (no. 342), one deletion (no. 565), and two cases of nucleotide substitution in exon 4 were shown in Figure 28. Figure 29 shows one duplication (no. 460) and two insertions (no. 105 and 219) in exon 4. Figure 30 shows one case with G to A base substitution in exons 5 (no. 514) and two cases of deletion (no.016 and 519) within intron 5. Several different mobility patterns of shifted bands were observed. The remaining cases did not have a mobility shift seen by SSCP analysis.

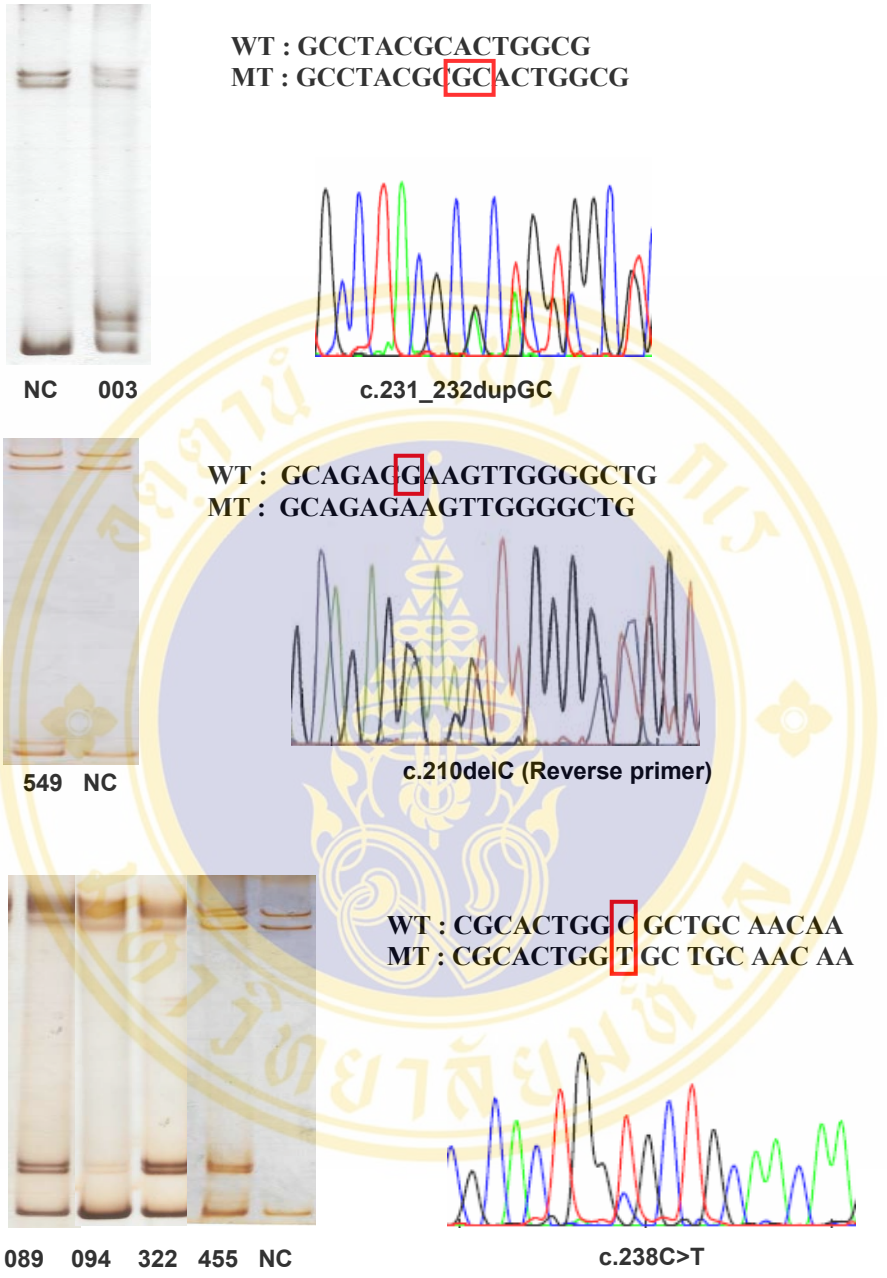


Figure 27 SSCP and sequencing analysis of AML patients with *AML1* mutation in exon 3. No. 003 and 549 were found to have duplication and deletion, respectively. C to A base substitution was found in patients no. 089, 094, 322, and 455. No.549 was sequenced using reverse primer. NC was negative control, WT was wild-type and MT was mutant.

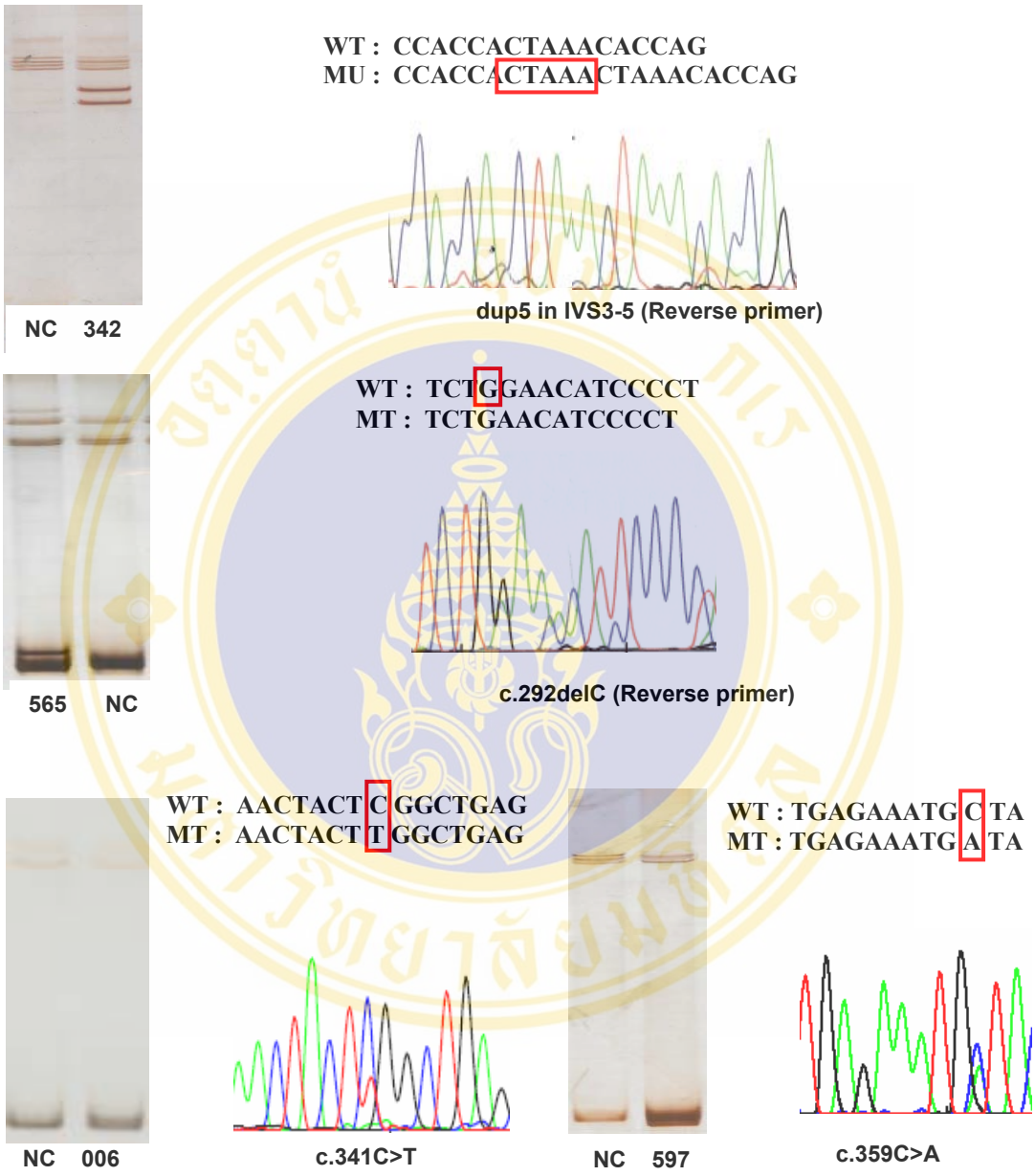


Figure 28 SSCP and sequencing analysis of AML patients with *AML1* mutation in intron 3 and exon 4. A duplication of 5 nucleotides in intron 3 was detected (no. 342). The deletion of one nucleotide and two cases with C to T or C to A base substitution were found in exon 4. Nucleotide sequences of no. 342 and 565 were sequenced by selected only mutated *AML1* allele from SSCP shifted band using reverse primer. NC was negative control, WT was wild-type and MT was mutant.

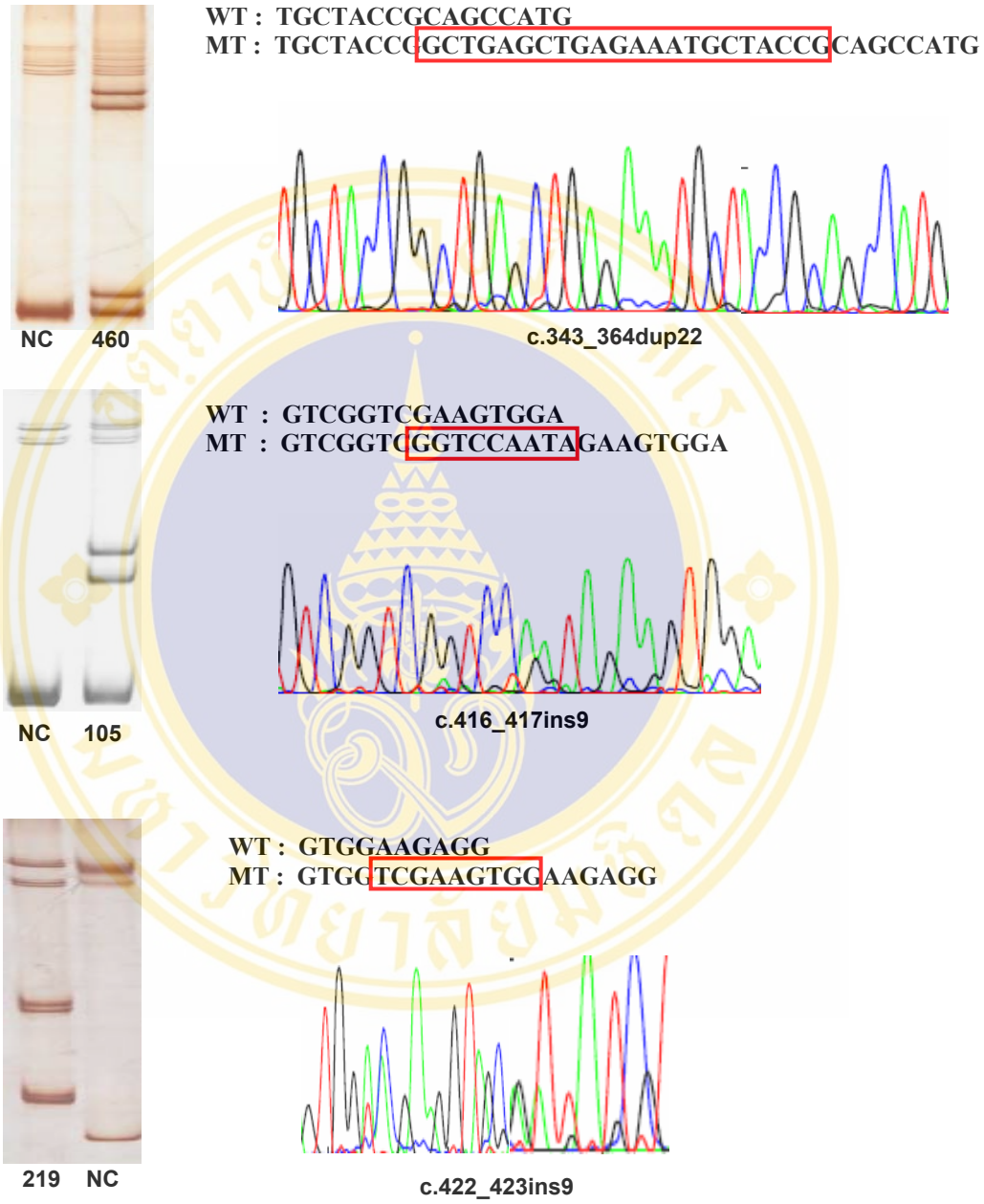


Figure 29 SSCP and sequencing analysis of AML patients with *AML1* mutation in exon 4. A duplication of 22 nucleotides and two insertions of 9 nucleotides within exon 4 were detected. Nucleotide sequences of no. 460 were sequenced by selected only mutated *AML1* allele from SSCP shifted band using reverse primer. NC was negative control, WT was wild-type and MT was mutant.

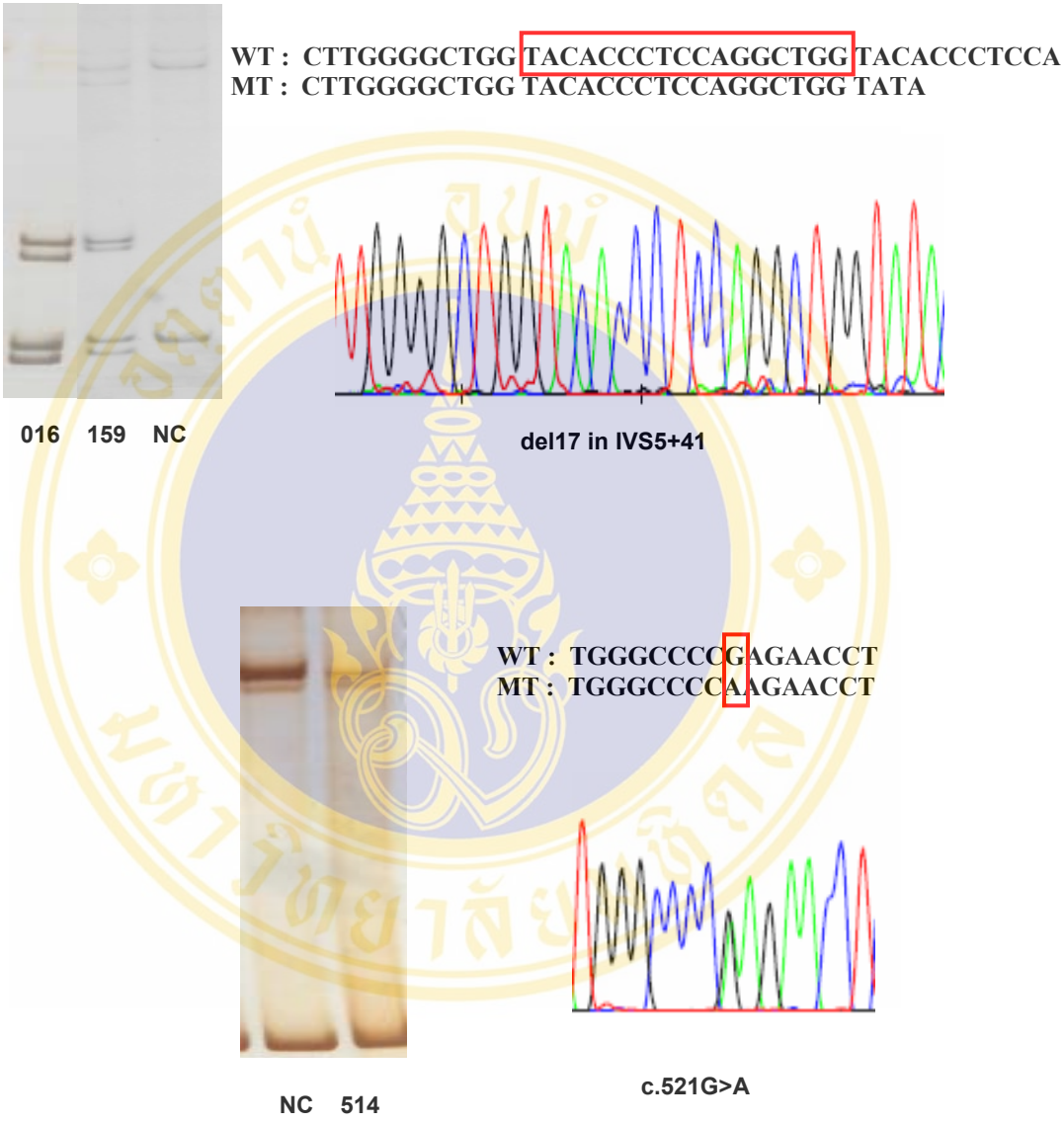


Figure 30 SSCP and sequencing analysis of AML patients with *AML1* mutation in exon 5 and intron 5. A deletion of 17 nucleotides in intron 5 (no. 016 and 159) and G to A base substitution of exon 5 (no. 514) were detected. Nucleotide sequences of no. 16 and 159 were sequenced by selected only mutated *AML1* allele from SSCP shifted band. NC was negative control, WT was wild-type and MT was mutant.

2.5 The locations and types of *AML1* mutation

Sixteen Thai adult AML cases had *AML1* mutations. Twelve mutations were found, including four known and eight novel mutations as shown in Table 16. The locations of *AML1* mutations were analyzed according to the nucleotide sequences of *AML1b* cDNA, GenBank accession no. D43968 using new nomenclature (104). Four of five patients with known mutations had the mutation at c.238C>T (no.089, 094, 322, and 455) and the other one at c.521G>A (no.514). In addition, two known mutations were found, including c.343_364dup22 (no. 460) and c.210delC (no. 549). Eight novel mutations were found including one of exon 3, five of exon 4, and one each of intron 3 and intron 5. The novel mutation of exon 3 was c.231_232dupGC (no. 003) which generated an immature stop codon at codon 94. Five novel mutations of exon 4 were c.341C>T (no. 006), c.416_417insGTCCAATAG (no. 105), c.422_423insTCGAAGTGG (no. 219), c.292delC (no. 565) and c.359C>A (no. 597). Additionally, one dupTTTAG in IVS3-5 (no. 342) and del17 in IVS5+41 (no. 016 and 159) were also found. The missense mutations including c.238C>T, c.341C>T c.359C>A and c.521G>A resulted in arginine changing to cysteine, serine to leucine, alanine to aspartic acid, and arginine to glutamine, respectively. Three insertions were found including the insertion of "GTCCAATAG" nucleotides at c.416_417 (case no. 105) which led to the insertion of serine, asparagine and arginine and the insertion of "TCGAAGTGG" nucleotides at c.422_423 that caused the addition of arginine, serine and glycine (case no. 219). Additionally, case no. 460 had a duplicated "GCTGAGCTGAGAAATGCTACCG" at codon 122 and caused the frameshift mutation which generated a stop codon at codon 123. Our study also found a novel duplication of "TTTAG" in intron 3 (dupTTTAG in IVS3-5) in a patient who also had a del(5q) by karyotype. This alteration inserted a new splicing acceptor site. The novel deletions were found in three AML cases including two cases with a 17-bp deletion of "TACACCCTCCAGGCTGG" in intron 5 (del17 in IVS5+41) and one with a deletion of one nucleotide base at codon 98 (c.292delC) (no. 565). Additionally, one case with a known deletion at codon 70 (c.210delC) was also found. All cases of *AML1* mutations were heterozygous because each patient could be shown to have one mutated allele and one normal allele.

Table 16 The location and type of 16 AML patients with *AML1* mutation.

No.	Type of mutation	*AML1* mutation		Exon
		cDNA	Predicted Protein	
003	Frameshift	c.231_2326dupGC[a]	T77fsX94	Exon 3
089	Missense	c.238C>T	R80C	Exon 3
094	Missense	c.238C>T	R80C	Exon 3
322	Missense	c.238C>T	R80C	Exon 3
455	Missense	c.238C>T	R80C	Exon 3
342	Frameshift	dup5 in IVS3-5[a]	Splicing defect	Intron 3
549	Frameshift	c.210delC	L71fsX94	Exon 3
006	Missense	c.341C>T[a]	S114L	Exon 4
105	Insertion	c.416_417insGTCCAATAG[a]	R139_S140insSNR	Exon 4
219	Insertion	c.422_423insTCGAAGTGG[a]	G141_R142insRSG	Exon 4
460	Frameshift	c.343_364dup22	A122fsX123	Exon 4
565	Frameshift	c.292delC[a]	P98fsX105	Exon 4
597	Missense	c.359C>A[a]	A120D	Exon 4
514	Missense	c.521G>A	R174Q	Exon 5
016	Deletion	del 17 in IVS5+41[a]	Polymorphism	Intron 5
159	Deletion	del 17 in IVS5+41[a]	Polymorphism	Intron 5

[a] novel mutation

2.6 The correlation of *AML1* mutation with FAB subtypes

Table 17 shows the frequency of *AML1* mutation in various AML subtypes according to FAB classification. Three of 98 (3.1%) M1, 2/114 (1.8%) M2, 3/78 (3.8%) M3, 4/67 (6%) M4, 2/32 (6.3%) M5, and 2/8 (28.6%) of M6 cases had *AML1* mutation. No *AML1* mutation was found in M0 and M7. The majority of Thai AML patients with *AML1* mutation were categorized as M4, M5, or M6 subtype. Fifty two normal healthy donors were also screened for *AML1* mutation in exons 3-5 and no one was found to carry the mutation or polymorphism.

Table 17 The frequency of *AML1* mutation identified in Thai AML patients (n=414).

AML subtype	No. studied	*AML1* mutation	%
M0	7	0	0
M1	98	3	3.1
M2	114	2	1.8
M3	78	3	3.8
M4	67	4	6
M5	32	2	6.25
M6	8	2	25
M7	1	0	0
Total	**414**	**16**	**4**

2.7 Clinical characteristics of AML patients with *AML1* mutation

The main features of 16 patients who were found to have either known (5 cases) or novel (11 cases) *AML1* mutation were summarized in Table 18. The majority of patients were young to middle-aged adults with the median age of 42 years. Four cases were older than 60 years old and seven patients were younger than 25 years old. The WBC counts were mostly below $50x10^9$/L, except for four patients. The Hb and Hct were low with the median of 8.2 g/dL and 25%, respectively.

Figure 31 shows the morphologic features of BM from AML-M4 and M6 patients who were found to harbor *AML1* mutation.

Table 18 Clinical and laboratory features of 16 AML patients with *AML1* mutation.

No.	AML subtype	Age	Hb	Hct	WBC	Plt
003	AML-M4	18/M	9.8	31	272	112
089	AML-M4	21/M	3.6	12	19.6	10
094	AML-M5	59/F	7.2	22	31	93
322	AML-M1	71/M	8.9	29	72	124
455	AML-M2	58/M	6.7	21	36.7	3
549	AML-M3	64/M	N/A	N/A	N/A	N/A
342	AML-M1	78/M	8.8	28	9.29	27
006	AML-M4	40/M	5.7	18	39.7	110
105	AML-M4	53/F	4.7	15	168	22
219	AML-M6	24/F	13.5	38	5.07	57
460	AML-M1	19/M	9	27	37.5	15
565	AML-M3	70/M	8.1	28	12.8	231
597	AML-M2	17/M	9.2	27	2.91	28
514	AML-M3	72/M	9.7	29.4	27.66	9
016	AML-M5	17/F	10.3	31	154	87
159	AML-M6	36/F	7	21	7.78	112

A. **B.**

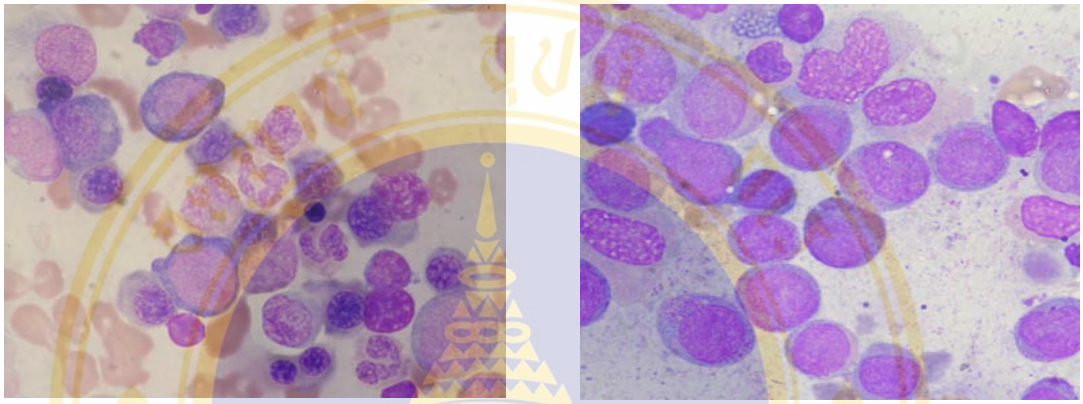


Figure 31 Morphological features of selected AML patients harboring *AML1* mutation. (A) and (B) were morphologic features of BM in patients no. 219 and 514, respectively.

2.8 Association of *AML1* mutation with chromosomal abnormalities

Among 16 patients with *AML1* mutation, the chromosomal data was available in 15 cases as summarized in Table 19. Normal karyotype was found in 7 cases. Eight of 15 patients (53%) had an abnormal karyotype. Trisomy 8 was found in 2 cases of patients with *AML1* mutation. Three cases had chromosome 21 abnormalities, including one case each of t(8;21) or t(6;21), and one case of 21q+ coexisted with monosomy 7. Additionally, one each of patient with *AML1* mutation had deletion of chromosome 5 or 9 and one case had monosomy 7. None of 16 patients with *AML1* mutations had other types of recurrent balanced translocations as classified by the recent WHO Classification, i.e. 11q23, t(15;17), inv(16) or the Philadelphia chromosome (t(9;22)).

Table 19 Chromosome abnormalities in AML patients with *AML1* mutations.

No.	AML subtype	Karyotype	*FLT3* genotype
003	AML-M4	46,XY	ITD
089	AML-M4	45,XY, -7,21q+	None
094	AML-M5	46,XX	ITD & TKD
322	AML-M1	46,XY /47,XY,+8	ITD
455	AML-M2	46,XY	None
549	AML-M3	N/A	None
342	AML-M1	46,XY, del(5)(q13q22)	None
006	AML-M4	46,XY	ITD
105	AML-M4	46,XX, del(9)(q13q34.2)	None
219	AML-M6	46,XX	None
460	AML-M1	46,XY, t(16;21)(p11.2;q22)	None
565	AML-M3	45,XY, -7	None
597	AML-M2	46,XY, t(8;21)(q22;q22)	None
514	AML-M3	47,XY, +8	None
016	AML-M5	46,XX	None
159	AML-M6	46,XX	None

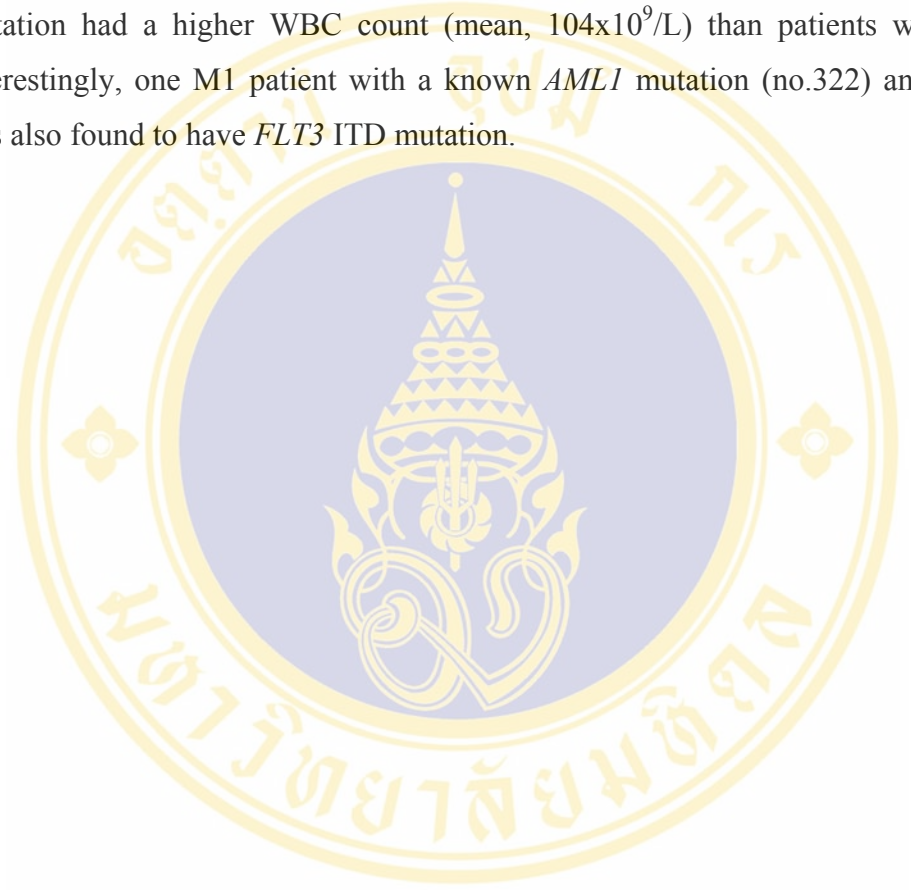
Table 20 shows the frequency of *AML1* mutation in each type of chromosomal abnormality including 2.6% (1/39) of AML patients with t(8;21), 33.3% (1/3) of patients with t(16;21) and 9.1% (1/7) of patients with trisomy 21 or 21q+. Additionally, 11.1% (1/9) patients with deletion 9, 15.4% (2/13) of patients with trisomy 8 and 12.5% of patients with either monosomy 5 or 7 had *AML1* mutation. Overall, 3 of 49 patients (6%) with chromosome 21 abnormalities had *AML1* mutation.

Table 20 The association of chromosome abnormalities and *AML1* mutation in 414 Thai adult AML patients**.**

Karyotype	**No. of studied**	***AML1* mutation**	**%**
t(8 ;21)	39	1	2.6
+21/21q+	7	1	9.1
t(16;21)	3	1	33.3
-5	8	1	12.5
-7	16	2	12.5
del(9)	9	1	11.1
+8	13	2	15.4
Normal karyotype	195	7	3.6

2.9 Association of *AML1* mutation with *FLT3* mutation

Four of 16 patients with *AML1* mutation (25%) had coexisting *FLT3* mutations as shown in Table 19. The eletrophoresis profiles of four cases with *FLT3* ITD mutation (no.003, 006, 094, and 322), and one case with TKD mutation (no.094) are shown in Figure 32. Patients with *AML1* mutation who had concomitant *FLT3* mutation had a higher WBC count (mean, $104x10^9$/L) than patients who did not. Interestingly, one M1 patient with a known *AML1* mutation (no.322) and trisomy 8 was also found to have *FLT3* ITD mutation.



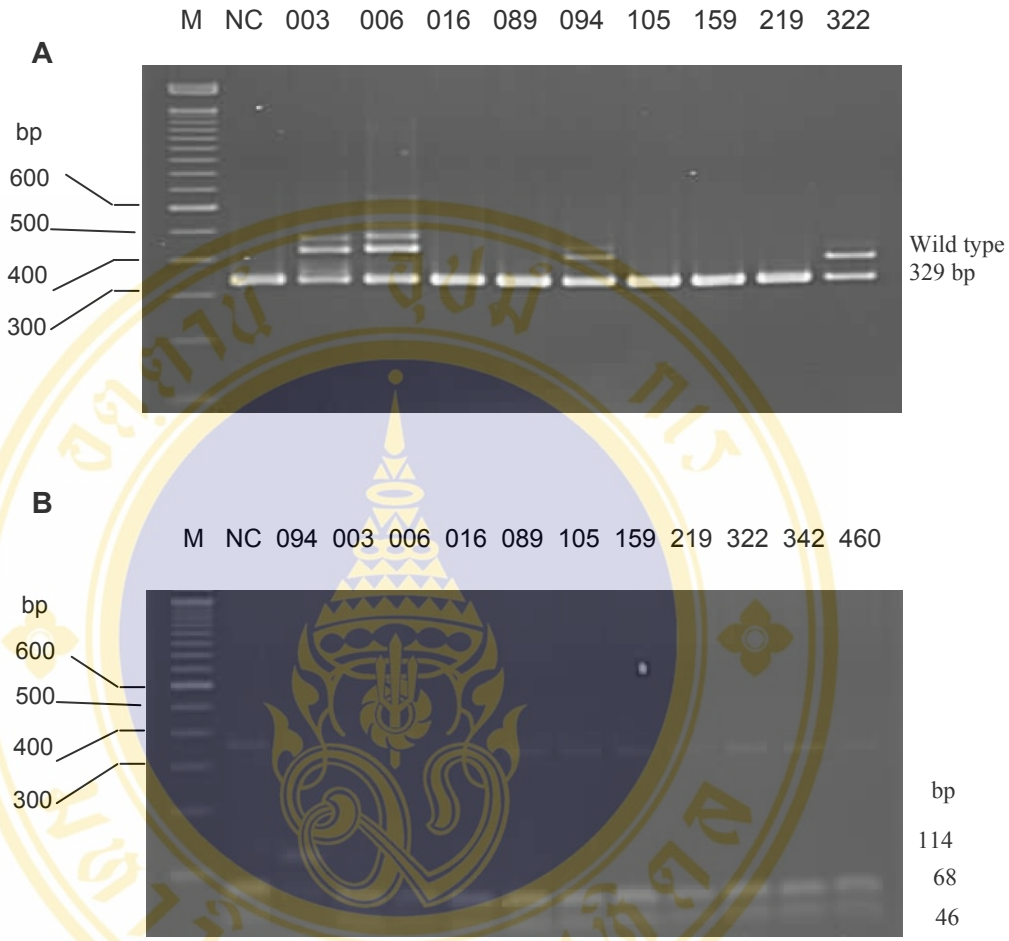


Figure 32 Agarose gel electrophoresis of AML patients with *AML1* mutation coexisted *FLT3* mutation. (A) AML patients with *AML1* mutation coexisted *FLT3-ITD* mutation. The aberrant ITD products identified as the higher molecular weight band than the wild type (no. 003, 006, 094, and 322). (B) AML patients with *AML1* mutation coexisted *FLT3-TKD* mutation (no.094). Lane NC is a negative control. Lane M is a 100-bp DNA ladder used as a standard-size marker.

CHAPTER VI
DISCUSSION

AML1 gene belongs to a class of transcription factor genes is frequently deregulated in leukemia, mainly through three mechanisms, i.e. translocation, point mutation and amplification. Although the first two mechanisms which are *AML1-ETO* gene rearrangement and *AML1* point mutations were predominantly found in AML and MDS cases, the last mechanism related to *AML1* gene amplification was uniquely reported only in patients with ALL (13). In this study, the two major mechanisms of *AML1* gene deregulation, i.e. translocation and point mutation were explored in a large cohort of Thai adult with *de novo* AML patients.

With respect to the translocation mechanism involving *AML1* gene in AML patients, RT-PCR-based assay was developed to detect the *AML1-ETO* fusion gene. The frequency of *AML1-ETO* fusion gene was found to be 15% among all AML cases and the frequency was proportionally higher in AML-M2 subtype (39%). The *AML1-ETO* fusion gene was also found in rare cases of M4 (6%) and M5 cases (13%) by morphology and flow cytometry. These results are in agreement with the frequency of *AML1-ETO* fusion gene reported in the literature as shown in Table 21. In the Japanese series, the frequency of *AML1-ETO* was between 15-18% of AML cases and 30-40% of AML-M2 subtype (6, 61). In Europe and North America, the incidence of *AML1-ETO* fusion gene was also similar to this present study, i.e. 7-15% of all AML subtypes and 18-40% in AML-M2 although a lower frequency of 6.8% of all AML subtypes was reported from the Brazilian series from South America (105). In Southeast Asia, only data from Malaysia was available (106). The frequency of *AML1-ETO* was 16.7% of AML cases and 40% of AML-M2 subtype. In Australia, the incidence of *AML1-ETO* was reported to be 7.4 % of AML and 40% of AML-M2 subtype (80).

Table 21 The frequency of t(8;21) or *AML1-ETO* in AML patients reported from various countries.

Countries	% t(8;21) in AML (all subtypes combined)	% t(8;21) in AML-M2
Asia		
Japan (6, 61)	15-18	30-40
Malaysia (106)	16.7	40
This study	15	39
North America and Europe		
USA (74)	7-15	18-40
France (72, 79)	8-20	30-40
UK (117, 118)	7-12.9	30.8-37
Denmark ((110)	4.3	N/A
Italy (109)	15	40
Sweden (112)	N/A	40
Germany (76, 113)	12-13	N/A
South America		
Brazil (105)	6.8	N/A
Australia (80**)**	7.4	40

N/A not available

The clinical and hematologic characteristics of AML-M2 patients with and without *AML1-ETO* were analyzed. Patients with *AML1-ETO* fusion gene were relatively younger with the median age of only 36 years as compared to 43 years ($p=0.006$) and had relatively lower WBC counts (median, $20.3x10^9$/L) than patients without the gene (median, $26x10^9$/L) but not statistically significant. Previous Japanese series also reported a difference of the WBC counts between the two groups, but age difference was not found (114). In the French and Canadian series, the *AML1-ETO* gene was also correlated with the young mean age of 24.3 years (72, 115).

To identify the correlation between the immunophenotypes and *AML1-ETO* fusion gene, the expression profiles of each myeloid and lymphoid antigenic marker on leukemic blasts based on the selected CD45 and side scatter gates were compared. It was found that AML–M2 patients with *AML1-ETO* displayed a significantly higher expression of CD56 and CD117 ($p<0.05$) than those without. Previous immunophenotypic analysis in the Canadian population have demonstrated that AML patients with *AML1-ETO* (n=18) had a relatively higher expression of CD34, TdT, CD19, CD56, and MPO but had a lower CD33 expression than the control group (n=387) (115). The French AML-M1 and M2 series also showed the correlation of CD56 but did not find the difference in CD33 expression between the groups with or without the fusion gene (72). The Italian series showed the higher expression of CD19, CD34, CD56, CD45RA, and CD54 but lower expression of CD45RO, CD33, CD36, CD11b and CD14 between patients with *AML1-ETO* (n=14) and control group (n=79) (109). One Japanese series reported the higher expression of CD34 and CD19 with lower CD33 expression in the *AML1-ETO*-positive group (114). Although the expression of each marker appeared to be somewhat different among each ethnic population, the expression of CD56 antigen in *AML1-ETO* positive patients was significantly higher than patients without this fusion gene in every studied ethnic population. CD56 was a known adhesion molecule marker (116) and may thus play role in the extramedullary manifestation that occurred more frequently in AML-M2 (116). Higher expression of the CD56 antigen was also known to be associated with poor prognosis and outcome in patients with *AML1-ETO* and CD56 expression as contrast to patients without CD56 expression (116, 117).

The frequency of *AML1* mutation in AML was previously reported from Japan, France and the United Kingdom as summarized in Table 22. No data from other Western or Asian countries exists with respect to the incidence and type of *AML1* mutation, either in M0 or non-M0 subtypes of AML. This study represents the first large series of *AML1* mutation ever reported from the Southeast Asian region and the largest in Asia. As seen in Table 22, the overall incidence of *AML1* mutation in the French series (n=414) was 10%. The highest frequency (22%) was observed in M0 AML followed by 7% in M3, 3% each in M2 and M5, 2% in M4 and none in M1 and M6 AML cases (13). Twelve percentage of the selected cases of the British M0 AML had *AML1* mutation (82) whereas 16-50% of the Japanese M0 AML did (15, 86, 90, 91). One Japanese series (n=131) reported the overall incidence of *AML1* mutation of 4.7% with the highest frequency of 22% in M0 AML (15). No cases of M3, M4, M5, M6 or M7 in this particular Japanese series were found to have *AML1* mutation. In present study, the overall frequency of *AML1* mutation of 4% was lower than the European series but in a close range to the Japanese series. A high incidence of *AML1* mutation was found in M6 followed by M4 and M5 subtype in our series which is strikingly different from other previously reported series in the West and Japan. However, it should be noted that very few cases of AML patients in this series were identified as M0 and none were found to carry *AML1* mutation. Larger number of M0 AML cases will be needed to determine the true incidence of *AML1* mutation in Thai M0 AML. As no other reports previously described *AML1* mutation in M6 patients, our study represents the first series to report 2 cases of M6 AML with *AML1* mutation, both of whom carried a novel mutation.

Table 21 Incidence of *AML1* mutation in AML patients as reported from other countries.

Study	No. of total cases reported	Frequency of *AML1* mutation in all subtypes (%)	Frequency of *AML1* mutation in specific subtypes (%)							
			M0	M1	M2	M3	M4	M5	M6	M7
Europe										
France										
Preudhomme C et al (2000) (83)	131	8	22	0	7	0	0	0	0	0
Roumier C et al (2003) (13)	414	10	22	0	3	7	2	3.1	0	2.8
Roumier C et al (2003) (88)	59	N/A	27	N/A	N/A	N/A	N/A	N/A	N/A	N/A
United Kingdom										
Langabeer SE et al (2002) (82)	61	N/A	12	N/A	N/A	N/A	N/A	N/A	N/A	5
Asia										
Japan										
Osato M et al (1999) (86)	109	5	33	0	0	8	4	8.3	0	N/A
Taketani T et al (2003) (91)	100	6	50	14	0	0	6	0	0	0
Matsuno N et al (2003) (90)	51	N/A	16	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Harada H et al (2004) (15)	131	4.7	22	5	3.5	0	0	0	0	0
Nakao M et al (2004) (120)	58	1.7	0	0	7.7	0	0	0	0	0
Thailand										
This study	**414**	**4**	**0**	**3.1**	**1.8**	**3.8**	**6.0**	**6.25**	**25**	**0**

N/A; data not available

The crystal structure of the runt domain-CBFβ-DNA tertianary complex has been recently determined and provides a structural basis for the effects of mutations on the biologic function of the AML1 protein as shown in Figure 7, page 26. The N-terminal region (exons 3 through 5) of AML1 contains a highly evolutionary conserved "runt" domain of 128 amino acids (6). The runt domain is responsible for both DNA binding ability and heterodimerization with the β subunit of core binding factor complex. DNA binding is mediated by 3 loops called loop (βA'-B), loop (βE'-F), and C-terminal tail in the runt domain, and the amino acid residues (R80, K83, T84, R135, R139, G141, R142, G143, I168, T169, V170, D171, R174 and R177) that directly contact the DNA were determined (33, 37, 41). In this study, 13 AML patients had mutations clustered within the runt domain, whereas 1 mutation was clustered in intron 3 and two in intron 5. Two known mutations including R80C (no.089, 094, 322, and 455) and R174Q (no.514) were also detected. Previous study in Japan found these two mutations in the patients with *de novo* MDS/AML and/or FPD/AML pedigrees. The functional analysis by the Japanese group demonstrated that R80C and R174Q were mapped to the three loops of AML1 and lacked the DNA-binding potential (13, 84, 86).

A novel duplication of "TTTAG" at the end of intron 3 (dupTTTAG in IVS3-5) was also discovered. This alteration produced a new splicing acceptor site that may provide a splicing defect. Four out of 16 patients with *AML1* mutation generated frameshift mutation (no.003, 460, 549 and 565) and were predicted to produce a premature stop codon that lack C-terminal end of protein products. Seven patients with missense mutations within the runt domain were predicted to loss DNA binding ability.

A novel deletion of "TACACCCTCCAGGCTGG" within intron 5 (del17 in IVS5+41) could be due to a rare polymorphism because the deleted sequences were distant to the splicing site of both exons 5 and 6. However, no mutations or polymorphisms could be detected in 52 normal healthy donors screened for *AML1* mutation in this study.

With respect to karyotype analysis, correlation between *AML1* mutation and karyotype data was determined. In this study, a novel *AML1* mutation which was

A147D was also found in a patient with t(8;21)(q22;q22) by karyotypic analysis. The known *AML1* mutation at c.343_364dup22 was also found in a patient with (16;21)(p11.2;q22). This mutation potentially loses the DNA binding potential and also lacks the transactivation activity. Additionally, a known mutation (R107C) was found in a patient of 21q+. It is of interest that point mutation of *AML1* gene together with chromosome 21 structural abnormalities. These results suggest that the occurrence of the *AML1* point mutation might be an additional molecular defect that leads to leukemogenesis by its cooperation with chromosomel 21 structural abnormalities. *AML1* mutation was also found in 1 of 9 (11.1%) patients with a partial deletion of the long arm of chromosome 9, del(9q), and 2 of 13 (15.4%) patients with trisomy 8. No data has previously reported the presence of *AML1* mutations with del(9q). Interestingly, del(9q) has been shown to coexist frequently with t(8;21) and CML with t(9;22). In my study, one patient with *AML1-ETO* gene (6.25%) also had del(9q). This result showed a lower frequency of del(9q) than the previous studies from other countries (66, 108). In Germany, 7 of 51 patients with *AML1-ETO* (14%) had del(9q). The median overall survival of the patients with del(9q) was significantly shorter than in patients with only t(8;21) (118, 119). Given that chromosome 9 has been shown to have a known oncogene such as *ABL* tyrosine kinase gene, it could be speculated that the *ABL* gene may cooperate with *AML1* rearrangement in this subgroup of AML patients in order to initiate and propagate the leukemic clone in AML patients (119).

To further support the model of multi-step leukemogenesis in AML, I explored if the cooperative mutations between *FLT3* (Class I mutation) and *AML1* (Class II mutation) existed in AML patients. The frequency of *FLT3* mutation in this series of non-M0 (25%, 4 of 16) was lower than that of M0 cases as reported by another group (63%, 5 of 8). Although the precise mechanism that initiates and propagates the leukemic clone in M0 and non-M0 cases is not known, we speculate that some common cooperative mechanisms exist in a small subset of patients. However, a unique mechanism that determines the final phenotype of most non-M0 cases (other than M2 with t(8;21), M3 with t(15;17) and M4 with inv(16)/t(16;16)) remains to be characterized as half of non-M0 AML patients do not have specific chromosomes and

molecular markers. It would be of interest to study further the gene expression profile of leukemic cells of patients with *AML1* mutation together with or without *FLT3* mutation, in M0 and non-M0 phenotype to determine the set of genes that may be differentially expressed.

AML1-ETO and *FLT3* mutations were two of the most frequent genetic alterations in AML. *AML1-ETO* generated by t(8;21) contributes to leukemic transformation, but additional events are required for full leukemogenesis. In animal model, experimental data indicates that, *AML1-ETO* and *FLT3* mutations are both insufficient on their own to cause leukemia (121). This study also examined whether *FLT3* mutation could be the genetic event that is associated with patients harboring t(8;21). Of 16 AML patients with *AML1-ETO* who were analyzed for the occurrence of *FLT3* mutations, *FLT3*-TKD mutation was found in only 1 out of 16 AML patients with *AML1-ETO* (6.25%) and *FLT3*-ITD was present in none of the patients with *AML-ETO* gene. The coexistence of *AML1-ETO* and *FLT3* mutations in Thailand was much lower than the previous report in Germany (121). This group found *FLT3-ITD* in 8.1% and *FLT3-TKD* in 2.2 % of patients with *AML1-ETO* (121). Previous study in our laboratory appeared to indicate that *FLT3* was more likely to cooperate with other transcription factor gene, i.e. *PML-RARA* gene in APL cases (122). Interestingly, *C-KIT* which also belongs to the same family of RTK as *FLT3* has been found to occur at such a high frequency than *FLT3* in AML with *AML1-ETO* (67). This data suggests that the cooperative genetic events do not occur randomly and the combination of genetic events could be very specific for each leukemia subtype.

With respect to the techniques used in this study, cytogenetic analysis was found to be quite sensitive for the detection of *AML1-ETO* gene as all cases with t(8;21) had detectable *AML1-ETO* gene by RT-PCR. Only one AML-M2 patient with a normal karyotype was found to have *AML1-ETO*. This data indicated that the sensitivity of cytogenetic analysis is comparable to the respective molecular test. Although RT-PCR method may have a higher sensitivity in some cases, particular variants of t(8;21) such as t(8;10;21) or t(8;10) or other structural chromosome lesions could not be identified by RT-PCR.

In conclusion, molecular techniques for the detection of *AML1-ETO* and *AML1* mutation have now been established in Thailand. This data suggest that the *AML1-ETO* and *AML1* mutation detection could easily be incorporated into the routine assessment of AML patients as the assay required only PCR and gel electrophoresis. These techniques should be useful for further classification and risk-stratification of AML patients in Thailand.

CHAPTER VII
CONCLUSION

Two major *AML1* gene deregulation mechanisms were identified in the large cohort of Thai adult *de novo* AML patients in this study. Fifteen percentages of all AML patients were found to have the *AML1-ETO* fusion gene. The majority of *AML1-ETO*-positive AML cases identified by the RT-PCR assay were categorized as AML-FAB M2 by morphology and immunophenotypes. All patients with t(8;21) by karyotypic analysis had detectable *AML1-ETO* fusion gene by RT-PCR. The unique clinical characteristics of AML patients with *AML1-ETO* fusion gene were their relatively young age and low WBC counts at diagnosis, both of which appeared to confirm the favorable prognostic impact of *AML1-ETO*. With respect to the immunophenotypic markers, AML-M2 patients with *AML1-ETO* had a typical feature of myeloid leukemia with high CD34, CD117, CD33, CD13, and CD11c expression. Moreover, they also uniquely displayed a significantly higher expression of NK-cell marker, i.e. CD56, than those without *AML1-ETO*.

This study represents the largest series in Asia to study the incidence of *AML1* mutation and also the first series in the Southeast Asian region. Point mutation of *AML1* gene is a rare finding and was found in only 4% of 414 AML patients in this study. All *AML1* mutations were categorized as non-M0 subtypes, predominantly as AML-M4, M5 and M6, which was strikingly different from the predilection for M0 in the Western reports. Interestingly, 8 novel mutations in 9 Thai AML patients involving exon 3, exon 4, intron 3, and intron 5 were identified in this study. The most common mutation in Thai population was R80C of exon 3. Two novel mutations were also detected in two patients with AML-M6, the subtype of which had not been previously reported in the literature to harbor *AML1* mutation. The clinical significance and biological functions of these new mutations require further studies in a larger populationA model of multiple cooperative events in acute leukemia was

exercised in this study. *FLT3* gene was chosen because of its RTK activity which belongs to Class I gene mutation that could potentially cooperate with *AML1* transcription factor gene and because of its high occurrence in adult AML cases. In this study, *FLT3* mutation was rarely found in AML patients with *AML1-ETO* fusion gene with only one case carrying both *AML1-ETO* and *FLT3* mutation in this study. On the contrary, a higher frequency of *FLT3* mutation was found in AML cases with *AML1* point mutations. Several additional genetic abnormalities were also found to coexist in patients with *AML1* mutations, supporting the model of multiple cooperating events that are critical in the development of AML with *AML1* mutations. Further studies of other Class I mutations such as C-Kit should be undertaken in the near future to identify the unique genetic events that may be of importance in the pathogenesis of acute leukemia that arises in patients harboring *AML1-ETO* fusion gene.

The ability to rapidly detect *AML1-ETO* fusion gene by a molecular assay should help physicians to correctly diagnose a favorable AML subtype, i.e. AML-M2 in Thailand and hopefully lead to better and appropriate therapeutic intervention. Future development of new drugs that target the interaction between *AML1, AML1-ETO* and their co-repressors and co-activator complexes may be another venue for AML-M2 therapy in a way that is similar to the effect of all-trans-retinoic acid (ATRA) on *PML-RARa* fusion gene in AML-M3 patients. Moreover, small molecules that could potentially stabilize DNA binding might also have therapeutic efficacy in AML patients with *AML1* mutations, particularly in the cases with mutations associated with loss of DNA-binding ability. The specific inhibitors of the tyrosine kinase function such as *FLT3* inhibitors might also prove useful in a subset of AML carrying both *FLT3* and *AML1* gene mutations.

As the current chemotherapeutic regimens could cure only a minute fraction of adult AML patients, novel strategies based on leukemia gene-targeting approach should be actively explored. The present information of *AML1* and *FLT3* genetic abnormalities should thus provide an important platform to guide us how to use gene-targeting therapy in Thai patients with AML in the near future.

REFERENCES

1. Giles FJ, Keating A, Goldstone AH, Avivi I, Willman CL, Kantarjian HM. Acute myeloid leukemia. Hematology (Am Soc Hematol Educ Program). 2002:73-110.
2. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999 Sep 30;341(14):1051-62.
3. Stone RM, O'Donnell MR, Sekeres MA. Acute myeloid leukemia. Hematology (Am Soc Hematol Educ Program). 2004:98-117.
4. Gilliland DG, Jordan CT, Felix CA. The molecular basis of leukemia. Hematology (Am Soc Hematol Educ Program). 2004:80-97.
5. Rowley JD. The role of chromosome translocations in leukemogenesis. Semin Hematol. 1999 Oct;36(4 Suppl 7):59-72.
6. Kurokawa M, Hirai H. Role of AML1/Runx1 in the pathogenesis of hematological malignancies. Cancer Sci. 2003 Oct;94(10):841-6.
7. Friedman AD. Leukemogenesis by CBF oncoproteins. Leukemia. 1999 Dec;13(12):1932-42.
8. Lorsbach RB, Downing JR. The role of the AML1 transcription factor in leukemogenesis. Int J Hematol. 2001 Oct;74(3):258-65.
9. Hart SM, Foroni L. Core binding factor genes and human leukemia. Haematologica. 2002 Dec;87(12):1307-23.
10. Speck NA, Stacy T, Wang Q, North T, Gu TL, Miller J, et al. Core-binding factor: a central player in hematopoiesis and leukemia. Cancer Res. 1999 Apr1;59(7 Suppl):1789s-93s.
11. Asou N. The role of a Runt domain transcription factor AML1/RUNX1 in leukemogenesis and its clinical implications. Crit Rev Oncol Hematol. 2003 Feb;45(2):129-50.
12. Peterson LF, Zhang DE. The 8;21 translocation in leukemogenesis. Oncogene. 2004 May 24;23(24):4255-62.

13. Roumier C, Fenaux P, Lafage M, Imbert M, Eclache V, Preudhomme C. New mechanisms of AML1 gene alteration in hematological malignancies. Leukemia. 2003 Jan;17(1):9-16.
14. Osato M. Point mutations in the RUNX1/AML1 gene: another actor in RUNX leukemia. Oncogene. 2004 May 24;23(24):4284-96.
15. Harada H, Harada Y, Niimi H, Kyo T, Kimura A, Inaba T. High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. Blood. 2004 Mar 15;103(6):2316-24.
16. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002;3:179-98.
17. Dash A, Gilliland DG. Molecular genetics of acute myeloid leukaemia. Best Pract Res Clin Haematol. 2001 Mar;14(1):49-64.
18. Schessl C, Rawat VP, Cusan M, Deshpande A, Kohl TM, Rosten PM, et al. The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. J Clin Invest. 2005 Aug;115(8):2159-68.
19. Appelbaum FR, Rowe JM, Radich J, Dick JE. Acute myeloid leukemia. Hematology (Am Soc Hematol Educ Program). 2001:62-86.
20. Mitani K. [Molecular mechanisms in leukemogenesis]. Gan To Kagaku Ryoho. 2002 Jul;29(7):1107-12.
21. Saglio G. Minimal residual disease detection in human leukemias: biologic and clinical significance. Acta Haematol Pol. 1995;26(2 Suppl 1):19-24.
22. Gale RP, Butturini A. Perspective: Chernobyl and leukemia. Leukemia. 1991 May;5(5):441-2.
23. Snyder R, Kalf GF. A perspective on benzene leukemogenesis. Crit Rev Toxicol. 1994;24(3):177-209.
24. Drabkin HA, Erickson P. Down syndrome and leukemia, an update. Prog Clin Biol Res. 1995;393:169-76.
25. Crombez EA, Dipple KM, Schimmenti LA, Rao N. Duplication of the Down syndrome critical region does not predict facial phenotype in a baby with a ring chromosome 21. Clin Dysmorphol. 2005 Oct;14(4):183-7.

26. Haferlach T, Schoch C. [WHO classification of acute myeloid leukaemia (AML) and the myelodysplastic syndrome (MDS)]. Dtsch Med Wochenschr. 2002 Mar 1;127(9):447-50.

27. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002 Oct 1;100(7):2292-302.

28. Klaus M, Haferlach T, Schnittger S, Kern W, Hiddemann W, Schoch C. Cytogenetic profile in de novo acute myeloid leukemia with FAB subtypes M0, M1, and M2: a study based on 652 cases analyzed with morphology, cytogenetics, and fluorescence in situ hybridization. Cancer Genet Cytogenet. 2004 Nov;155(1):47-56.

29. Rowley JD. The critical role of chromosome translocations in human leukemias. Annu Rev Genet. 1998;32:495-519.

30. Mitani K. Chromosomal abnormalities and oncogenes. Int J Hematol. 1996 Feb;63(2):81-93.

31. Hayashi Y. The molecular genetics of recurring chromosome abnormalities in acute myeloid leukemia. Semin Hematol. 2000 Oct;37(4):368-80.

32. Sawyers CL. Molecular abnormalities in myeloid leukemias and myelodysplastic syndromes. Leuk Res. 1998 Dec;22(12):1113-22.

33. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002 Jul;2(7):502-13.

34. Uchida H, Zhang J, Nimer SD. AML1A and AML1B can transactivate the human IL-3 promoter. J Immunol. 1997 Mar 1;158(5):2251-8.

35. Shimizu K, Kitabayashi I, Kamada N, Abe T, Maseki N, Suzukawa K, et al. AML1-MTG8 leukemic protein induces the expression of granulocyte colony-stimulating factor (G-CSF) receptor through the up-regulation of CCAAT/enhancer binding protein epsilon. Blood. 2000 Jul 1;96(1):288-96.

36. Miyoshi H, Ohira M, Shimizu K, Mitani K, Hirai H, Imai T, et al. Alternative splicing and genomic structure of the AML1 gene involved in acute myeloid leukemia. Nucleic Acids Res. 1995 Jul 25;23(14):2762-9.

37. Tahirov TH, Inoue-Bungo T, Morii H, Fujikawa A, Sasaki M, Kimura K, et al. Structural analyses of DNA recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFbeta. Cell. 2001 Mar 9;104(5):755-67.

38. Nucifora G, Rowley JD. The AML1 gene in the 8;21 and 3;21 translocations in chronic and acute myeloid leukemia. Cold Spring Harb Symp Quant Biol. 1994;59:595-605.

39. Lo Coco F, Pisegna S, Diverio D. The AML1 gene: a transcription factor involved in the pathogenesis of myeloid and lymphoid leukemias. Haematologica. 1997 May-Jun;82(3):364-70.

40. Werner MH, Shigesada K, Ito Y. Runt domains take the lead in hematopoiesis and osteogenesis. Nat Med. 1999 Dec;5(12):1356-7.

41. Blyth K, Cameron ER, Neil JC. The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer. 2005 May;5(5):376-87.

42. Perry C, Eldor A, Soreq H. Runx1/AML1 in leukemia: disrupted association with diverse protein partners. Leuk Res. 2002 Mar;26(3):221-8.

43. Hiebert SW, Downing JR, Lenny N, Meyers S. Transcriptional regulation by the t(8;21) fusion protein, AML-1/ETO. Curr Top Microbiol Immunol. 1996;211:253-8.

44. Douet-Guilbert N, Morel F, Le Bris MJ, Herry A, Le Calvez G, Marion V, et al. Translocation (12;21) followed by insertion of chromosome 3 material in the derivative chromosome 12 in a case of childhood acute lymphoblastic leukemia. Cancer Genet Cytogenet. 2003 Apr 15;142(2):120-3.

45. Raynaud S, Cave H, Baens M, Bastard C, Cacheux V, Grosgeorge J, et al. The 12;21 translocation involving TEL and deletion of the other TEL allele: two frequently associated alterations found in childhood acute lymphoblastic leukemia. Blood. 1996 Apr 1;87(7):2891-9.

46. Nucifora G, Begy CR, Erickson P, Drabkin HA, Rowley JD. The 3;21 translocation in myelodysplasia results in a fusion transcript between the AML1 gene and the gene for EAP, a highly conserved protein associated with the Epstein-Barr virus small RNA EBER 1. Proc Natl Acad Sci U S A. 1993 Aug 15;90(16):7784-8.

47. Nucifora G, Rowley JD. The AML1 and ETO genes in acute myeloid leukemia with a t(8;21). Leuk Lymphoma. 1994 Aug;14(5-6):353-62.

48. Lenny N, Meyers S, Hiebert SW. Functional domains of the t(8;21) fusion protein, AML-1/ETO. Oncogene. 1995 Nov 2;11(9):1761-9.

49. Kozu T, Miyoshi H, Shimizu K, Maseki N, Kaneko Y, Asou H, et al. Junctions of the AML1/MTG8(ETO) fusion are constant in t(8;21) acute myeloid leukemia detected by reverse transcription polymerase chain reaction. Blood. 1993 Aug 15;82(4):1270-6.

50. Erickson P, Gao J, Chang KS, Look T, Whisenant E, Raimondi S, et al. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood. 1992 Oct 1;80(7):1825-31.

51. Hug BA, Lazar MA. ETO interacting proteins. Oncogene. 2004 May 24;23(24):4270-4.

52. Davis JN, McGhee L, Meyers S. The ETO (MTG8) gene family. Gene. 2003 Jan 16;303:1-10.

53. Gamou T, Kitamura E, Hosoda F, Shimizu K, Shinohara K, Hayashi Y, et al. The partner gene of AML1 in t(16;21) myeloid malignancies is a novel member of the MTG8(ETO) family. Blood. 1998 Jun 1;91(11):4028-37.

54. Morohoshi F, Mitani S, Mitsuhashi N, Kitabayashi I, Takahashi E, Suzuki M, et al. Structure and expression pattern of a human MTG8/ETO family gene, MTGR1. Gene. 2000 Jan 11;241(2):287-95.

55. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci U S A. 1991 Dec 1;88(23):10431-4.

56. Wolford JK, Prochazka M. Structure and expression of the human MTG8/ETO gene. Gene. 1998 May 28;212(1):103-9.

57. Amann JM, Nip J, Strom DK, Lutterbach B, Harada H, Lenny N, et al. ETO, a target of t(8;21) in acute leukemia, makes distinct contacts with multiple histone deacetylases and binds mSin3A through its oligomerization domain. Mol Cell Biol. 2001 Oct;21(19):6470-83.

58. Downing JR. The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: biology and clinical significance. Br J Haematol. 1999 Aug;106(2):296-308.

59. Sakakura C, Yamaguchi-Iwai Y, Satake M, Bae SC, Takahashi A, Ogawa E, et al. Growth inhibition and induction of differentiation of t(8;21) acute myeloid leukemia cells by the DNA-binding domain of PEBP2 and the AML1/MTG8 (ETO)-specific antisense oligonucleotide. Proc Natl Acad Sci U S A. 1994 Nov 22;91(24):11723-7.

60. Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc Natl Acad Sci U S A. 1998 Sep 1;95(18):10860-5.

61. Okuda T, Cai Z, Yang S, Lenny N, Lyu CJ, van Deursen JM, et al. Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. Blood. 1998 May 1;91(9):3134-43.

62. Drescher B, Nagel S, Krauter J, Heidenreich O, Ganser A, Heil G. AML1/ETO inhibits AML1/CCAAT-enhancer binding protein-alpha mediated activation of the CD11c promoter and represses CD11c expression in HL60 cells. Haematologica. 2003 Aug;88(8):956-8.

63. Wildonger J, Mann RS. The t(8;21) translocation converts AML1 into a constitutive transcriptional repressor. Development. 2005 May;132(10):2263-72.

64. Rhoades KL, Hetherington CJ, Harakawa N, Yergeau DA, Zhou L, Liu LQ, et al. Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. Blood. 2000 Sep 15;96(6):2108-15.

65. Fenske TS, Pengue G, Graubert TA. Dominant negative effects of the AML1/ETO fusion oncoprotein. Cell Cycle. 2005 Jan;4(1):33-6.

66. Sweetser DA, Peniket AJ, Haaland C, Blomberg AA, Zhang Y, Zaidi ST, et al. Delineation of the minimal commonly deleted segment and identification of candidate tumor-suppressor genes in del(9q) acute myeloid leukemia. Genes Chromosomes Cancer. 2005 Nov;44(3):279-91.

67. Wang YY, Zhou GB, Yin T, Chen B, Shi JY, Liang WX, et al. AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. Proc Natl Acad Sci U S A. 2005 Jan 25;102(4):1104-9.

68. Yuan Y, Zhou L, Miyamoto T, Iwasaki H, Harakawa N, Hetherington CJ, et al. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. Proc Natl Acad Sci U S A. 2001 Aug 28;98(18):10398-403.

69. Frank R, Zhang J, Uchida H, Meyers S, Hiebert SW, Nimer SD. The AML1/ETO fusion protein blocks transactivation of the GM-CSF promoter by AML1B. Oncogene. 1995 Dec 21;11(12):2667-74.

70. Ferrara F, Del Vecchio L. Acute myeloid leukemia with t(8;21)/AML1/ETO: a distinct biological and clinical entity. Haematologica. 2002 Mar;87(3):306-19.

71. Downing JR, Higuchi M, Lenny N, Yeoh AE. Alterations of the AML1 transcription factor in human leukemia. Semin Cell Dev Biol. 2000 Oct;11(5):347-60.

72. Andrieu V, Radford-Weiss I, Troussard X, Chane C, Valensi F, Guesnu M, et al. Molecular detection of t(8;21)/AML1-ETO in AML M1/M2: correlation with cytogenetics, morphology and immunophenotype. Br J Haematol. 1996 Mar;92(4):855-65.

73. Rege K, Swansbury GJ, Atra AA, Horton C, Min T, Dainton MG, et al. Disease features in acute myeloid leukemia with t(8;21)(q22;q22). Influence of age, secondary karyotype abnormalities, CD19 status, and extramedullary leukemia on survival. Leuk Lymphoma. 2000 Dec;40(1-2):67-77.

74. Mrozek K, Prior TW, Edwards C, Marcucci G, Carroll AJ, Snyder PJ, et al. Comparison of cytogenetic and molecular genetic detection of t(8;21) and inv(16) in a prospective series of adults with de novo acute myeloid leukemia: a Cancer and Leukemia Group B Study. J Clin Oncol. 2001 May 1;19(9):2482-92.

75. Cox MC, Panetta P, Venditti A, Del Poeta G, Franchi A, Buccisano F, et al. Comparison between conventional banding analysis and FISH screening

with an AML-specific set of probes in 260 patients. Hematol J. 2003;4(4):263-70.

76. Wattjes MP, Krauter J, Nagel S, Heidenreich O, Ganser A, Heil G. Comparison of nested competitive RT-PCR and real-time RT-PCR for the detection and quantification of AML1/MTG8 fusion transcripts in t(8;21) positive acute myelogenous leukemia. Leukemia. 2000 Feb;14(2):329-35.

77. Barragan E, Bolufer P, Moreno I, Martin G, Nomdedeu J, Brunet S, et al. Quantitative detection of AML1-ETO rearrangement by real-time RT-PCR using fluorescently labeled probes. Leuk Lymphoma. 2001 Aug;42(4):747-56.

78. Maruyama F, Stass SA, Estey EH, Cork A, Hirano M, Ino T, et al. Detection of AML1/ETO fusion transcript as a tool for diagnosing t(8;21) positive acute myelogenous leukemia. Leukemia. 1994 Jan;8(1):40-5.

79. Morschhauser F, Cayuela JM, Martini S, Baruchel A, Rousselot P, Socie G, et al. Evaluation of minimal residual disease using reverse-transcription polymerase chain reaction in t(8;21) acute myeloid leukemia: a multicenter study of 51 patients. J Clin Oncol. 2000 Feb;18(4):788-94.

80. Mitterbauer M, Kusec R, Schwarzinger I, Haas OA, Lechner K, Jaeger U. Comparison of karyotype analysis and RT-PCR for AML1/ETO in 204 unselected patients with AML. Ann Hematol. 1998 Mar-Apr;76(3-4):139-43.

81. Steer EJ, Goldman JM, Cross NC. Mutations of the transcription factor AML1/CBFA2 are uncommon in blastic transformation of chronic myeloid leukaemia. Leukemia. 2001 Mar;15(3):476-7.

82. Langabeer SE, Gale RE, Rollinson SJ, Morgan GJ, Linch DC. Mutations of the AML1 gene in acute myeloid leukemia of FAB types M0 and M7. Genes Chromosomes Cancer. 2002 May;34(1):24-32.

83. Preudhomme C, Warot-Loze D, Roumier C, Grardel-Duflos N, Garand R, Lai JL, et al. High incidence of biallelic point mutations in the Runt domain of the AML1/PEBP2 alpha B gene in Mo acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21. Blood. 2000 Oct 15;96(8):2862-9.

84. Song WJ, Sullivan MG, Legare RD, Hutchings S, Tan X, Kufrin D, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat Genet. 1999 Oct;23(2):166-75.

85. Steensma DP, Gibbons RJ, Mesa RA, Tefferi A, Higgs DR. Somatic point mutations in RUNX1/CBFA2/AML1 are common in high-risk myelodysplastic syndrome, but not in myelofibrosis with myeloid metaplasia. Eur J Haematol. 2005 Jan;74(1):47-53.

86. Osato M, Asou N, Abdalla E, Hoshino K, Yamasaki H, Okubo T, et al. Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2alphaB gene associated with myeloblastic leukemias. Blood. 1999 Mar 15;93(6):1817-24.

87. Imai Y, Kurokawa M, Izutsu K, Hangaishi A, Takeuchi K, Maki K, et al. Mutations of the AML1 gene in myelodysplastic syndrome and their functional implications in leukemogenesis. Blood. 2000 Nov 1;96(9):3154-60.

88. Roumier C, Eclache V, Imbert M, Davi F, MacIntyre E, Garand R, et al. M0 AML, clinical and biologic features of the disease, including AML1 gene mutations: a report of 59 cases by the Groupe Francais d'Hematologie Cellulaire (GFHC) and the Groupe Francais de Cytogenetique Hematologique (GFCH). Blood. 2003 Feb 15;101(4):1277-83.

89. Carnicer MJ, Nomdedeu JF, Lasa A, Estivill C, Brunet S, Aventin A, et al. FLT3 mutations are associated with other molecular lesions in AML. Leuk Res. 2004 Jan;28(1):19-23.

90. Matsuno N, Osato M, Yamashita N, Yanagida M, Nanri T, Fukushima T, et al. Dual mutations in the AML1 and FLT3 genes are associated with leukemogenesis in acute myeloblastic leukemia of the M0 subtype. Leukemia. 2003 Dec;17(12):2492-9.

91. Taketani T, Taki T, Takita J, Tsuchida M, Hanada R, Hongo T, et al. AML1/RUNX1 mutations are infrequent, but related to AML-M0, acquired trisomy 21, and leukemic transformation in pediatric hematologic malignancies. Genes Chromosomes Cancer. 2003 Sep;38(1):1-7.

92. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations of AML1 are common in therapy-related myelodysplasia following therapy with alkylating agents and are significantly associated with deletion or loss of chromosome arm 7q and with subsequent leukemic transformation. Blood. 2004 Sep 1;104(5):1474-81.

93. Humphries SE, Gudnason V, Whittall R, Day IN. Single-strand conformation polymorphism analysis with high throughput modifications, and its use in mutation detection in familial hypercholesterolemia. The IFCC Scientific Division: Committee on Molecular Biology Techniques. J Int Fed Clin Chem. 1997 Dec;9(4):156-61.

94. Sunnucks P, Wilson AC, Beheregaray LB, Zenger K, French J, Taylor AC. SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. Mol Ecol. 2000 Nov;9(11):1699-710.

95. Miterski B, Kruger R, Wintermeyer P, Epplen JT. PCR/SSCP detects reliably and efficiently DNA sequence variations in large scale screening projects. Comb Chem High Throughput Screen. 2000 Jun;3(3):211-8.

96. Busson-Le Coniat M, Nguyen Khac F, Daniel MT, Bernard OA, Berger R. Chromosome 21 abnormalities with AML1 amplification in acute lymphoblastic leukemia. Genes Chromosomes Cancer. 2001 Nov;32(3):244-9.

97. Ferro MT, Hernaez R, Sordo MT, Garcia-Sagredo JM, Garcia-Miguel P, Fernandez Guijarro M, et al. Chromosome 21 tandem repetition and AML1 (RUNX1) gene amplification. Cancer Genet Cytogenet. 2004 Feb;149(1):11-6.

98. Gilliland DG, Griffin JD. Role of FLT3 in leukemia. Curr Opin Hematol. 2002 Jul;9(4):274-81.

99. Bernasconi P, Boni M, Cavigliano PM, Calatroni S, Giardini I, Rocca B, et al. Molecularly targeted therapy in acute myeloid leukemia. Ann N Y Acad Sci. 2004 Dec;1028:409-22.

100. Auewarakul CU, Promsuwicha O, Y UP, Pattanapanyasat K, Issaragrisil S. Immunophenotypic profile of adult acute myeloid leukemia (AML):

analysis of 267 cases in Thailand. Asian Pac J Allergy Immunol. 2003 Sep;21(3):153-60.

101. Auewarakul CU, Sritana N, Limwongse C, Thongnoppakhun W, Yenchitsomanus PT. Mutations of the FLT3 gene in adult acute myeloid leukemia: determination of incidence and identification of a novel mutation in a Thai population. Cancer Genet Cytogenet. 2005 Oct 15;162(2):127-34.
102. Udomsakdi-Auewarakul C**,** Promsuwicha O, Tochaoroentanaphol C, Munkhetvit C, Pattanapanyasat K, Issaragrisil S. Immunophenotypes and outcome of Philadelphia chromosome (Ph^1)-positive and negative Thai adult acute lymphoid leukemia. Int J Hematol 2003; 78: 337-43.
103. Mitelman F, ed. ISCN 1995: An International System for Human Cytogenetic Nomenclature 1995. Basel, Switzerland: S Karger, 1995.
104. Den Dunnen JT, Antonarakis SE. Nomenclature for the description of human sequence variations. Hum Genet. 2001 Jul;109(1):121-4.
105. Mendes WL, Coser VM, Ramos G, Pereira W, Lopes LF, de Oliveira MS. The apparent excess of acute promyelocytic leukemia in infant acute leukemias in Brazil. Haematologica. 2004 Nov;89(11):ELT16.
106. Rosline H, Narazah M. Y, Illunihayati I, Isa M N, Baba AA. The Detection of AML1/ETO Fusion Transcript in Acute Myeloid Leukaemia in Universiti Sains Malaysia Hospital. AsPac J. Mol. Biol. Biotechnol., 2004;12:49-52.
107. Rowe D, Cotterill SJ, Ross FM, Bunyan DJ, Vickers SJ, Bryon J, et al. Cytogenetically cryptic AML1-ETO and CBF beta-MYH11 gene rearrangements: incidence in 412 cases of acute myeloid leukaemia. Br J Haematol. 2000 Dec;111(4):1051-6.
108. Tobal K, Yin JA. Monitoring of minimal residual disease by quantitative reverse transcriptase-polymerase chain reaction for AML1-MTG8 transcripts in AML-M2 with t(8; 21). Blood. 1996 Nov 15;88(10):3704-9.
109. Langabeer SE, Walker H, Rogers JR, Burnett AK, Wheatley K, Swirsky D, et al. Incidence of AML1/ETO fusion transcripts in patients entered into the MRC AML trials. MRC Adult Leukaemia Working Party. Br J Haematol. 1997 Dec;99(4):925-8.

110. Pallisgaard N, Hokland P, Riishoj DC, Pedersen B, Jorgensen P. Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. Blood. 1998 Jul 15;92(2):574-88.

111. Ferrara F, Di Noto R, Annunziata M, Copia C, Lo Pardo C, Boccuni P, et al. Immunophenotypic analysis enables the correct prediction of t(8;21) in acute myeloid leukaemia. Br J Haematol. 1998 Jul;102(2):444-8.

112. Mitelman F, Heim S. Quantitative acute leukemia cytogenetics. Genes Chromosomes Cancer. 1992 Jul;5(1):57-66.

113. Schnittger S, Weisser M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFBMYH11+ acute myeloid leukemia based on quantification of fusion transcripts. Blood. 2003 Oct 15;102(8):2746-55.

114. Kita K, Nakase K, Miwa H, Masuya M, Nishii K, Morita N, et al. Phenotypical characteristics of acute myelocytic leukemia associated with the t(8;21)(q22;q22) chromosomal abnormality: frequent expression of immature B-cell antigen CD19 together with stem cell antigen CD34. Blood. 1992 Jul 15;80(2):470-7.

115. Khoury H, Dalal BI, Nevill TJ, Horsman DE, Barnett MJ, Shepherd JD, et al. Acute myelogenous leukemia with t(8;21)--identification of a specific immunophenotype. Leuk Lymphoma. 2003 Oct;44(10):1713-8.

116. Baer MR, Stewart CC, Lawrence D, Arthur DC, Byrd JC, Davey FR, et al. Expression of the neural cell adhesion molecule CD56 is associated with short remission duration and survival in acute myeloid leukemia with t(8;21)(q22;q22). Blood. 1997 Aug 15;90(4):1643-8.

117. Raspadori D, Damiani D, Lenoci M, Rondelli D, Testoni N, Nardi G, et al. CD56 antigenic expression in acute myeloid leukemia identifies patients with poor clinical prognosis. Leukemia. 2001 Aug;15(8):1161-4.

118. Schoch C, Haase D, Haferlach T, Gudat H, Buchner T, Freund M, et al. Fifty-one patients with acute myeloid leukemia and translocation t(8;21)(q22;q22): an additional deletion in 9q is an adverse prognostic factor. Leukemia. 1996 Aug;10(8):1288-95.

119. Marcucci G, Mrozek K, Ruppert AS, Maharry K, Kolitz JE, Moore JO, et al. Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): a Cancer and Leukemia Group B study. J Clin Oncol. 2005 Aug 20;23(24):5705-17.

120. Nakao M, Horiike S, Fukushima-Nakase Y, Nishimura M, et al. Novel loss-of-function mutations of the haematopoiesis-related transcription factor, acute myeloid leukaemia 1/runt-related transcription factor 1, detected in acute myeloblastic leukaemia and myelodysplastic syndrome. Br J Haematol 2004; 125:709-19.

121. Schessl C, Rawat VP, Cusan M, Deshpande A, Kohl TM, Rosten PM, et al. The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. J Clin Invest. 2005 Aug;115(8):2159-68.

122. Le Beau MM, Davis EM, Patel B, Phan VT, Sohal J, Kogan SC. Recurring chromosomal abnormalities in leukemia in PML-RARA transgenic mice identify cooperating events and genetic pathways to acute promyelocytic leukemia. Blood. 2003 Aug 1;102(3):1072-4.

APPENDIX

APPENDIX A

List of chemicals, instruments, enzyme, DNA markers, and reagents

1. Chemicals

Chemicals	Molecular weight (g/ml)	Source
Absolute ethanol (C_2H_5OH)	46.07	E.Merck, Darmstadtt, F.A., Germany.
Absolute methanol (CH_3OH)	32.04	E.Merck, Darmstadtt, F.A., Germany.
Acrylamide (C_3H_5NO)	71.08	Bio Basic Inc., Toronto, Canada.
Agarose ($(C_{12}H_{18}O_9)_n$)		Seakem, Rockland, USA.
Ammonium Chloride (NH_4Cl)	53.49	Sigma chemical, St, Louis, USA.
Ammonium persulphate ($(NH_4)_2S_2O_8$	228.20	Sigma chemical, St, Louis, USA.
Boric Acid (H_3BO_3)	61.83	USB, Cleveland, USA.
Bromophenol blue ($C_{19}H_9Br_4O_5SNa$)	670.00	Promega, Madison, USA.
Chloroform ($CHCl_3$)	119.38	E.Merck, Darmstadtt, F.A., Germany.
Citric acid ($CHCl_3$)	210.14	Fissher Scientific, Leics, UK.
3′-Deoxyadenosine 5’-triphosphate or dATP ($C_{10}H_{12}N_5O_{12}P_3Na_4$)	579.2	Promega, Madison, USA.
3′-Deoxycytosine 5’-triphosphate or dCTP ($C_9H_{12}N_3O_{13}P_3Na_4$)	555.1	Promega, Madison, USA.

3′-Deoxyguanosine 5’-triphosphate or dGTP ($C_{10}H_{12}N_5O_{13}P_3Na_4$)	595.1	Promega, Madison, USA.
3′-Deoxythymidine 5’-triphosphate or dTTP ($C_{10}H_{13}N_2O_{14}P_3Na_4$)	570.1	Promega, Madison, USA
Ethidium bromide	394.3	Research Organics, Cleveland, USA.
Ethylenediamine tetra acetic acid tetrasodium salt or EDTA ($C_{10}H_{12}N_2Na_2O_8$)	380.2	E.Merck, Darmstadtt, F.A., Germany.
Ficoll (Type 400)	400,000	Sigma chemical, St, Louis, USA.
Formaldehyde solution (Formalin) (CH_2O)	30.2	A.N.H Products Ltd., Bangkok, Thailand.
Glycerol ($C_3H_8O_3$)	92.09	BDH Laboratory Supplies Poole, England.
Hydrochloric acid (HCl)	36.50	E.Merck, Darmstadtt, F.A., Germany.
Isoamyl alcohol ($C_5H_{12}O$)	88.15	E.Merck, Darmstadtt, F.A., Germany.
Isopropanol ($CH_3CHOHCH_3$)	60.10	E.Merck, Darmstadtt, F.A., Germany.
N,N’-Methylene-bis-acrylamide($C_7H_{10}N_2O_2$)	154.20	Sigma chemical, St, Louis, USA.
Nitric acid (HNO_3)	63.01	E.Merck, Darmstadtt, F.A., Germany.
Phenol (C_6H_5OH)	94.11	E.Merck, Darmstadtt, F.A., Germany.
Potassium chloride (KCl)	74.56	E.Merck, Darmstadtt, F.A., Germany.
Potassium hydrogen carbonate ($KHCO_3$)	100.12	E.Merck, Darmstadtt, F.A., Germany.
Silver nitrate ($AgNO_3$)	169.87	E.Merck, Darmstadtt, F.A.,

		Germany.
Sodium acetate (CH_3COONa)	82.03	USB, Cleveland, USA.
Sodium carbonate (Na_2CO_3)	105.99	E.Merck, Darmstadtt, F.A., Germany.
Sodium chloride (NaCl)	58.44	E.Merck, Darmstadtt, F.A., Germany.
Sodium dodecyl sulphate or SDS ($C_{12}H_{25}O_4SNa$)	288.38	Bio Basic Inc., Toronto, Canada.
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	137.99	E.Merck, Darmstadtt, F.A., Germany.
Sodium hydrogen phosphate (Na_2HPO_4)	141.96	E.Merck, Darmstadtt, F.A., Germany.
Sodium hydroxide (NaOH)	40.00	E.Merck, Darmstadtt, F.A., Germany.
N, N, N', N'-Tetramethyl ethylenediamine or TEMED ($C_4H_{11}NO_3$)	166.21	Bio-Rad Laboratories, Hong Kong.
Tris (Hydroxymethyl aminomethane) ($NH_2C(CH_2OH)_3$)	121.14	USB, Cleveland, USA.
Xylene cyanol FF ($C_{25}H_{27}N_2O_6Na$)	538.60	Bio Basic Inc., Toronto, Canada.

2. Instruments

Applied Biosystems 377 DNA sequencer, Applied Biosystems, Foster, USA.
Gel documentation system, UVItec, Cambridge, UK.
GeneAmp PCR system 2400, Applied Biosystems, Foster, USA.
GeneAmp PCR system 9600, Applied Biosystems, Foster, USA.
Horizontal gel electrophoresis apparatus, ATTO corporation, Tokyo, Japan.
Mini gel electrophoresis apparatus, Labnet international Inc., Edision, USA.
Power supplies, E143, 200-400 V, CONSORT, Turnhout, Belgium.
UV-transilluminator, FOTODYNE incorporated, Hartland, USA.
UV-visible spectrophotometer, Shimadzu UV-160A, Kyoto, Japan.

3. Enzymes

Proteinase K, Promega, Madison, USA.
Taq DNA polymerase, Promega, Madison, USA.
Inmolase *Taq* DNA polymerase, Bioline, Germany

4. DNA markers

100 bp DNA Ladder, New England BioLabs Inc., Beverly, USA.

5. Reagents

5.1 10x Phosphate buffered saline (PBS)

NaCl	40	g
KCl	1	g
Na_2HPO_4	0.1	g
KH_2PO_4	1	g
Distilled water to	500	ml

The solution was autoclaved for 15 minutes at 121 °C, 15 lb/ inches^2 and stored at room temperature. It was diluted to 1x before use. (1x PBS consists of 137 mM NaCl, 2.7 KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH~7.3).

5.2 10x RBC lysis buffer

NH_4Cl	0.829	g
$KHCO_3$	0.1	g
EDTA	0.0037	g
Distilled water to	100	ml

The solution was autoclaved for 15 minutes at 121 °C, 15 lb/inches^2 and stored at room temperature.

5.3 70% Ethanol

Absolute ethanol	70	ml
Sterile distilled water to	100	ml

The solution was mixed and stored at –20 °C.

5.4 10 mM Tris-HCl, pH 8.0

Tris base	12.11	g

Dissolved in distilled water and adjusted pH 8 with HCl

Added distilled water to	100	ml

The solution was sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches and stored at room temperature.

5.5 0.5 M EDTA pH 8.0

EDTA	18.60	g

Dissolved in distilled water and adjusted pH to 8.0 with NaOH

Added distilled water to	100	ml

The solution was mixed and stored at room temperature

5.6 TE 20-5 (20mM Tris-HCl and 5 mM EDTA) buffer

20 mM Tris –HCl, pH 8.0	1	volume
5 mM EDTA	1	volume

The solution was sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches and stored at room temperature.

5.7 50x Tris-acetate buffer (TAE buffer)

Tris-base (Sigma)	242	g
Glacial acetic acid	57.1	ml
0.5 M EDTA (pH 8.0)	100	ml
Added distilled water to	1000	ml

The solution was mixed and stored at room temperature. It was diluted to 1x before use (1x TAE consist of 0.04 M Tris-acetate and 0.001 M EDTA).

5.8 5x Tris-borate buffer (TBE buffer)

Tris-base (Sigma)	54	g
Boric acid	27.5	g
0.5 M EDTA (pH 8.0)	20	ml
Added distilled water to	1000	ml

The solution was mixed and stored at room temperature.

5.9 10% (w/v) SDS solution

Sodium dodecyl sulfate	10	g
Added distilled water to	100	ml

The solution was sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches and stored at room temperature.

5.10 2 mg/ml proteinase K in TE 20-5

Proteinase K	2	mg
Sterile TE 20-5 buffer	1000	µl

The solution was mixed and immediately used.

5.11 Phenol (equilibrated several times with 0.1 m Tris-HCl pH 8.0 and added 8-hydroxyquinoline to 0.1% w/v)

Liquified phenol	1	volume

Added 8-Hydroxyquinoline to a final conc. 0.1%

The solution was mixed with equal volume of buffer (0.1 M Tris-HCl, pH 8.0) and melted at 68 °C. Then the upper (aqueous) phase was removed until the pH of phenolic phase is >7.8

Added β-Mercaptoethanol to a final conc. 0.2%

The phenol solution was mixed and stored in a light tight bottle at 4 °C for periods of up to 1 month.

5.12 4 M NaCl solution

NaCl	23.38	g
Added distilled water to	100	ml

The solution is dispensed into aliquots and autoclaved.

5.13 Chloroform-isoamyl alcohol (24:1)

Chloroform	24	volume
Isoamyl alcohol	1	volume

The reagents were mixed and stored in the sterile bottle at room temperature.

5.14 mM dNTPs solution (containing 2 mM dATP, 2 mM dCTP, 2 mM dGTP, 2 mM dTTP), to prepare 100 µl, mix

100 mM dATP (Promega)	2	µl
100 mM dTTP (Promega)	2	µl
100 mM dCTP (Promega)	2	µl
100 mM dGTP (Promega)	2	µl
DEPC-treated water	92	µl

The solution was kept at -20°C.

1.3% (w/v) Agarose gel in 1x TAE buffer

Agarose gel	1.3	g
1x TAE buffer	100	ml

The slurry was heated in microwave oven until the agarose completely dissolved and then poured the warm agarose solution into the gel box. The gel was between 3 mm and 5 mm thick. It was checked to ensure that there were no air bubbles under or between the teeth of the comb.

5.15 6x gel loading buffer

0.25%	bromophenol blue
0.25%	xylene cyanol
15%	Ficoll (Type 400; Sigma) in water

The reagents were mixed them together and stored in aliquots at 4 °C.

5.16 Sample running buffer for single stranded DNA

Formamide	950	µl
5% bromophenol blue	10	µl
5% xylene cyanol	10	µl
1 M EDTA	20	µl
1 M NaOH	10	µl

The buffer was aliquoted and stored at 4 °C.

5.17 40% acrylamide:bisacrylamide (49:1) stock solution

Acrylamide	39.2	g
N, N'-methylenebisacrylamide	0.8	g
Deionized water	~80	ml

The solution was dissolved at 65 °C for 10 minutes, filtered through 0.45 mm filter and adjusted volume to 100 ml with deionized water and kept at 4°C.

5.18 10% (w/v) Ammonium persulphate (APS)

Ammonium persulphate	1.0	g
Added distilled water to	10	ml

The solution was kept in the vial at 4 °C.

5.19 40% Methanol

Methanol	400	ml
Added deionized water to	1000	ml

The solution was mixed and stored at room temperature.

5.20 160 mM Nitric acid (HNO_3)

70% HNO_3	10.19	ml
Added deionized water to	1000	ml

The solution was mixed and stored at room temperature.

5.21 Silver staining solution

Silver nitrate ($AgNO_3$)	100	mg
Added deionized water to	50	ml

The solution was mixed and used immediately.

5.22 Developer solution

Sodium carbonate (Na_2CO_3)	2.1	g
Added deionized water to	70	ml
35% Formaldehyde	35	µl

The solution was mixed and used immediately.

5.23 Stop solution

Citric acid	100	g
Added deionized water to	1000	ml

The solution was mixed and stored at room temperature.

5.24 3 M Sodium acetate (NaOAc), pH 4.6

Sodium acetate	16.40	g

Dissolved in distilled water and adjusted pH to 5.0 with glacial acetic acid

Added distilled water to 100 ml

The solution was mixed, and autoclaved for 15 minutes at 121 °C, 15 lb/square inches and stored at room temperature

APPENDIX B

Publication and presentations

List of publication from this thesis

1. Chirayu U Auewarakul, **Amporn Leecharendkeat**, Wanna Thongnoppakhun, Chanin Limwongse, Chintana Tocharoentanaphol. Mutations of *AML1* gene in non-M0 acute myeloid leukemia: discovery of six novel mutations with high incidence of cooperative events in Southeast Asian population. Hematologica. 2006. (In press)

List of presentations from this thesis

Oral presentation

1. **Amporn Leecharendkeat**, Wanna Thongnoppakhun, Chanin Limwongse, Pathai Yenchitsommanas, Chirayu U Auewarakul. Translocations and point mutations of *AML1* gene in Thai adult acute myeloid leukemia patients. Siriraj Medical Congress 2005 "Excellent Medical Practices for Better Quality of Life" , 4-8 July, 2005.

Poster presentation

1. Chirayu U Auewarakul, Orathai Promsuwicha**, Amporn Leecharendkeat**, Chintana Tocharoentanaphol. Analysis of chromosome aberrations that involved hemopoietic transcription factor gene, *AML1*, in Thai acute myeloid leukemia. The 5 th Princess Chulabhorn International Science Congress, 16-20 August 2004, Chulabhon Research Institute.

BIOGRAPHY

NAME	Amporn Leecharendkeat
DATE OF BIRTH	September 17, 1981
PLACE OF BIRTH	Chiangrai, Thailand
INSTITUTE ATTENDED	Chiangmai University, 1999-2002 Bachelor of Science (Medical Technology) Mahidol University, 2003-2005 Master of Science (Immunology)
RESEARCH GRANT	Supported in part by the Thesis Grant, Faculty of Graduate Studies, Mahidol University
SCHOLARSHIP	Mahidol University Graduate Student Alumni Association and the Faculty of Medicine Siriraj Hospital Graduate Studies
ADDRESS	177/120 Soi Wattana, Prannok road Bangkok noi, Bangkok.