ANALYSIS OF *PML/RARα* FUSION GENE VARIANTS AND IDENTIFICATION OF ASSOCIATED MEMBRANE PROTEIN MARKERS IN THAI PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA

ORATHAI PROMSUWICHA

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Orathai Promsuwicha

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ABSTRACT

The objectives of this research were 1) to determine the frequency and type of *PML/RARα* fusion gene variants (*bcr1, bcr2 or bcr 3*) in Thai adult acute promyelocytic leukemia (APL) patients, 2) to explore if specific immunophenotypic markers could be identified to predict the presence of promyelocytic leukemia/retinoic acid receptor-alpha (*PML/RARα*) fusion genes in leukemic samples from APL patients, and 3) to determine if additional genetic event, i.e., Fms-like tyrosine kinase (*FLT*)*-3* mutation, coexists in Thai APL patients.

Leukemic samples from 80 APL patients were analyzed by morphologic, immunophenotypic, karyotypic and molecular analysis. Flow cytometry was performed using a cocktail of monoclonal antibodies and CD45/side scatter selection gates. RNA and DNA were obtained from bone marrow or blood and the breakpoint sites of the *PML/RARα* fusion gene were identified by reverse transcription-polymerase chain reaction (RT-PCR) with a nested approach. A NB4 leukemic cell line was used as a positive control for *PML/RARα* gene. Genomic DNA was analyzed by standard polymerase chain reaction (PCR), to detect *FLT3*-ITD mutation. *FLT3*-TKD mutations in codon 835 and 836 were screened by RFLP analysis using an *Eco*RV enzyme.

The bcr1, bcr2, and bcr3 variants, were detected in 71.3%, 3.7%, and 2.5%, respectively, of APL patients diagnosed by flow cytometry. The majority of cytogenetically confirmed APL cases (81.3%) had bcr1 while 12.5% had bcr2 and 6.3% had bcr3. In APL patients without t(15;17), *PML/RARα* gene was also detected in 46 cases (57.5%). The immunophenotypes that were specific for APL with *PML/RARα* gene lacked HLA-DR and CD34 with high CD13, CD33, and MPO expression. The positive predictive value (PPV) and negative predictive value (NPV) of $CD34^-HLA\text{-}DR^-$ to predict APL with *PML/RARα* was 72.2% and 100%, respectively. The PPV for CD33, CD117, MPO and CD33 was 75.8, 93.8, 72.3 and 46.7%, respectively. No APL cases were positive for both CD34 and HLA-DR as compared to non-APL cases (n=529, $p<0.05$). The hematologic parameters of APL patients with bcr1 were similar to bcr2 and bcr3. Overall, APL cases were predominantly middle-aged females with the median age of 41 years. *FLT3* gene mutation was more frequently found in APL (38.8%) as contrast to non-APL cases with t(8;21)(15.2%, p=0.045), with the incidence of 26.3% and 12.5%, for ITD and TKD mutation in APL, respectively.

In conclusion, bcr1 is the most frequently detected *PML/RARα* gene variant in Thai APL patients. The specific immunophenotypic marker identified to predict the presence of *PML/RARα* gene was lack of CD34 and HLA-DR. *FLT3* mutation frequently coexisted in APL patients with *PML/RARα* gene. Molecular analysis of *PML/RARα* gene appears to be more sensitive than conventional karyotypic analysis for the confirmation of the diagnosis of APL. This study represents the first study in Thailand to report the incidence and type of *PML/RARα* gene variants and the coexistence of *FLT3* gene. The availability of the rapid nested RT-PCR assay to detect *PML/RARα* gene is very useful for confirming the diagnosis of APL by flow cytometry and justifying the prompt use of all-trans-retinoic acid therapy. The current knowledge of *FLT3* gene mutation in APL patients should be valuable for the future design of specific gene-targeted therapy that would hopefully lead to better overall survival and more cure of patients with this tragic disease.

KEY WORDS: ACUTE PROMYELOCYTIC LEUKEMIA / PML/RARα FUSION GENE VARIANT / FMS-LIKE TYROSINE KINASE 3 (FLT3) GENE

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การวิเคราะห์ชนิดของยีน *PML/RARα* และชนิดของโปรตีนบนผิวเซลล์มะเร็งเม็ดเลือดขาวเฉียบพลันชนิดโปรมัยอีโลซัยท์ในผู้ป่วยไทย (ANALYSIS OF *PML/RARα* FUSION GENE VARIANTS AND IDENTIFICATION OF ASSOCIATED MEMBRANE PROTEIN MARKERS IN THAI PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA)

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คณะกรรมการควบคุมวิทยานิพนธ์ : จิรายุ เอื้อวรากุล, พ.บ., Ph.D., โกวิท พัฒนาปัญญาสัตย์, Ph.D., เพทาย เย็นจิตโสมนัส, Ph.D., วรรณา ทองนพคุณ, Ph.D.

บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์เพื่อ 1) พัฒนาวิธีการตรวจยีนลูกผสม *PML/RARα* และศึกษาชนิดต่างๆ ของยีน *PML/RARα* (bcr1, bcr2 และ bcr3) ในคนไทย 2) ค้นหาแอนติเจนที่จำเพาะในการตรวจอิมมูโนฟีโนทัยป์เพื่อทำนายการเกิด *PML/RARα* ในผู้ป่วยมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดโปรมัยอีโลซัยท์ และ 3) ศึกษาอุบัติการณ์ของยีน *FLT3* ในผู้ป่วยมะเร็งเม็ดเลือดขาวชนิดโปรมัยอีโลซัยท์

ตัวอย่างตรวจไขกระดูกหรือเลือดของผู้ป่วยมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดโปรมัยอีโลซัยท์ จำนวน 80 รายที่ได้ผ่านการวินิจฉัยโดยการตรวจลักษณะเซลล์ อิมมูโนฟีโนทัยป์และการตรวจโครโมโซมตามวิธีมาตรฐาน ได้ถูกนำมาสกัดสารพันธุกรรมชนิดดีเอนเอและอาร์เอนเอ เพื่อใช้ในการตรวจหายีน *PML/RARα* โดยวิธี nested RT-PCR โดยมีเซลล์ไลน์ NB4 ที่มี t(15;17) เป็นตัวเปรียบเทียบ สำหรับการตรวจหามิวเตชันของ *FLT3* เป็น *FLT3*- ITD ใช้วิธีพีซีอาร์ ส่วนการตรวจหามิวเตชันของ *FLT3*-TKD ที่ตำแหน่ง codon 835 และ 836 ใช้วิธีย่อยด้วยเอนไซม์ตัดจำเพาะ *Eco*RV ตามด้วย RFLP

ผลการวิจัยพบสัดส่วนของยีนลูกผสม *PML/RARα* ในกลุ่มผู้ป่วย APL ที่วินิจฉัยด้วยอิมมูโนฟีโนทัยป์ ดังนี้ bcr1 71.3%, bcr2 3.7% และ bcr3 2.5% สำหรับผู้ป่วยที่มี t(15;17) จากการตรวจวิธีทางโครโมโซมพบ bcr1 81.3%, bcr2 12.5% และ bcr3 6.2% สำหรับผู้ป่วย APL ที่ไม่มีโครโมโซม t(15;17) พบ *PML/RARα* 46 ราย (57.5%) ลักษณะเฉพาะของ APL ที่มี t(15;17) และ/หรือ *PML/RARα* คือ ไม่พบการแสดงออกของแอนติเจน CD34 และ HLA-DR ร่วมกันโดยความสามารถในการทำนายโรคเมื่อผลทดสอบ (CD34-HLA-DR-) เป็นบวก เท่ากับ 72.2% และความสามารถในการทำนายโรคเมื่อผลทดสอบเป็นลบ เท่ากับ 100% สำหรับ CD13, CD117, MPO และ CD33 มีความสามารถในการทำนายโรคเมื่อผลทดสอบเป็นบวก เท่ากับ 75.8%, 93.8%, 72.3% และ 46.7% ตามลำดับ ไม่มีผู้ป่วย APL รายใดที่พบ CD34 และ HLA-DR ร่วมกันเมื่อเทียบกับผู้ป่วย AML ชนิดอื่นจำนวน 529 ราย ($p<0.05$) โดยรวมผู้ป่วย APL เป็นผู้หญิงและมีอายุเฉลี่ยอยู่ที่ 41 ปี จากการศึกษาพบมิวเตชันของยีน *FLT3* ในผู้ป่วย APL (38.8%) มากกว่าในผู้ป่วย AML ที่มี t(8;21) (15.2%, p=.0.045) ในผู้ป่วย APL พบ *FLT3* ชนิด ITD จำนวน 21 ราย (26.3%) และ TKD จำนวน 10 ราย (12.5%)

โดยสรุป การศึกษานี้ตรวจพบยีน *PML/RARα* ชนิด bcr1 มากที่สุดในผู้ป่วยไทยที่เป็นมะเร็งเม็ดเลือดขาวเฉียบพลันชนิด APL และเมื่อเปรียบเทียบกับผลจากการตรวจโครโมโซมวิธีปกติ พบว่าการตรวจหายีน *PML/RARα* โดยวิธี nested RT-PCR มีความไวมากกว่าโครโมโซมในการยืนยันการวินิจฉัย APL ระดับโมเลกุล และทำให้สามารถใช้ยากลุ่ม retinoic acid เพื่อรักษาผู้ป่วย APL ได้อย่างถูกต้องและเร็วขึ้น ลักษณะจำเพาะของการตรวจอิมมูโนฟีโนทัยป์ในการทำนายการเกิด *PML/RARα* คือ การตรวจไม่พบ CD34 และHLA-DR การวิจัยครั้งนี้ถือเป็นครั้งแรกที่ได้ทราบอุบัติการณ์และชนิดของ *PML/RARα* และยีน *FLT3* ในผู้ป่วยไทยที่เป็นAPL ซึ่งจะเป็นประโยชน์ต่อไปในการพิจารณาเลือกใช้ยาที่มุ่งเป้าที่ยีน *PML/RARα* และยีน *FLT3* เพื่อให้ผู้ป่วยมีโอกาสรอดชีวิตและหายขาดจากโรคมากขึ้น

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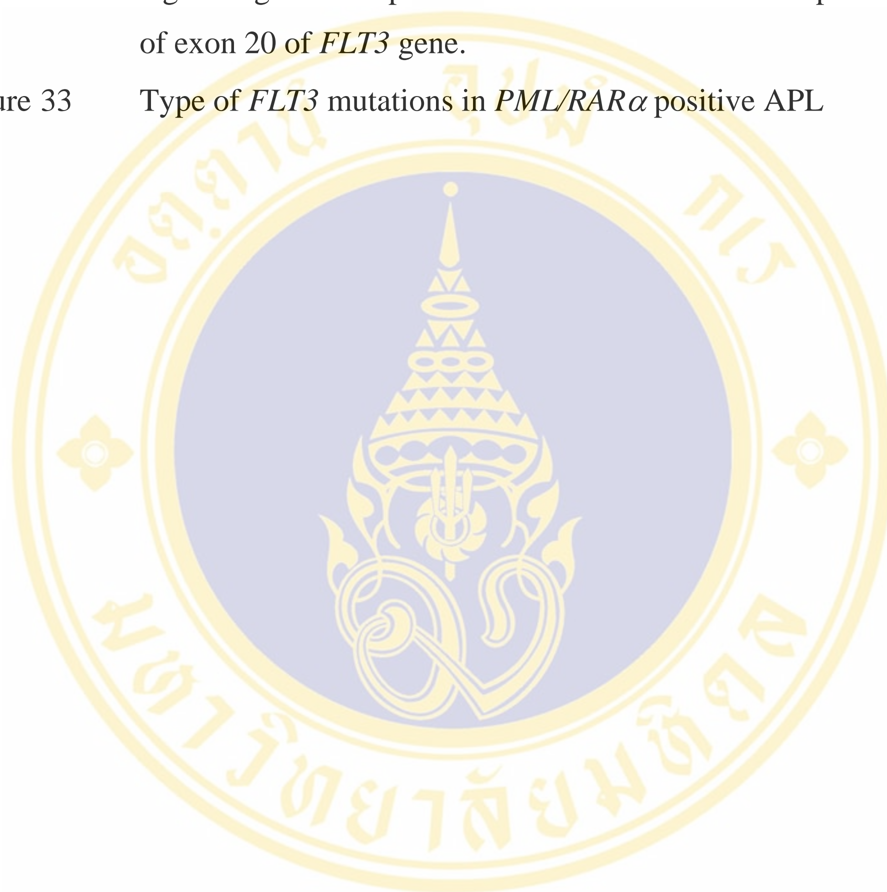

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LIST OF ABBREVIATIONS

Abbreviations		Term
ABL	=	Abelson murine leukemia viral oncogene
AML	=	acute myeloblastic leukemia
APL	=	acute promyelocytic leukemia
ATRA	=	all- *trans*-retinoic acid
bcr	=	break point cluster region
bp	=	base pair
°C	=	degree Celsius
dATP	=	deoxyadenosine-5'-triphosphate
dCTP	=	deoxycytosine-5'-triphosphate
DEPC	=	diethyl pyro-carbonate
dGTP	=	deoxyguanosine-5'-triphosphate
DNA	=	deoxyribonucleic acid
dTTP	=	deoxythymidine-5'-triphosphate
EDTA	=	ethylenediamine tetraacetic acid
ETO	=	eight twenty one
FISH	=	fluorescence in situ hybridization
FLT3	=	FMS-like tyrosine kinase 3
ITD	=	internal tandem duplication
M	=	molar
mg	=	milligram
ml	=	millilitre
mM	=	millimolar
µg	=	microgram
µl	=	microlitre

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LIST OF ABBREVIATIONS (cont.)

Abbreviations		Term
μM	=	micromolar
ng	=	nanogram
nm	=	nanomolar
nt	=	nucleotide
OD	=	optical density
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
PML	=	promyelocytic leukemia
pmol	=	picomolar
RARα	=	retinoic acid alpha
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription polymerase chain reaction
rpm	=	revolutions per minute
Taq	=	*Thermus aquaticus*
TE	=	Tris-EDTA buffer
TBE	=	Tris-borate EDTA buffer
Tm	=	melting temperature
TKD	=	tyrosine kinase domain
U	=	unit
UV	=	ultraviolet

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CHAPTER I

INTRODUCTION

Acute promyelocytic leukemia (APL) is a well-defined subtype of acute leukemia characterized by the t(15;17)(q22;q21) translocation. This translocation reflects the molecular rearrangement of the promyelocytic leukemia (PML) gene, located at 15q22, with the retinoic acid receptor alpha (RARα) gene, located at 17q21, and is considered to be critical for the pathogenesis of the disease since it blocks differentiation during the promyelocytic stage of myeloid maturation. The vitamin A derivative, all-*trans*-retinoic acid (ATRA) is the drug of first choice in the treatment of newly diagnosed APL. All patients who harbor *PML/RARα* transcripts respond to ATRA differentiation therapy. High concentrations of ATRA restore the gene expression and apoptosis program disrupted by the presence of *PML/RARα.*

Among APL cases with a *PML/RARα* rearrangement, chromosome 15 breakpoints fall within three breakpoint cluster regions (bcrs) that have been completely sequenced, although occasional patients have breakpoints outside these regions. Bcr1 and bcr3 correspond to *PML* introns 6 and 3 or 4, respectively, whereas bcr2 is located within *PML* exon 6. Bcr1 breakpoints result in an mRNA fusion of *PML* exon 6 to *RARα* exon 3, conventionally referred to as the L (long) isoform. Bcr2 breakpoints usually result in a tripartite mRNA fusion in which part of *PML* exon 6 is fused to an insert sequence and then to *RARα* exon 3. Bcr3 breakpoints result in an mRNA fusion of *PML* exon 3 or exon 4 to *RARα* exon 3, referred to as the S (short) isoform.

A leukemic stem cell model proposes that leukemic blasts originate from a common primitive progenitor that has the capacity to self-renew. The hematopoietic stem cell is typically defined by three distinct properties: self-renewal, differentiation

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capacity and proliferation capacity. For leukemia to arise, it is believed that at least two "hits" or mutations are required. One class of mutations, exemplified by *BCR-ABL* and *FLT3* mutations, confers proliferative and/or survival advantage to hematopoietic progenitors but has no effect on the differentiation in hematopoietic progenitors. In contrast, other class of mutations causing the loss of function of hematopoietic transcription factors result in a block in differentiation at a specific stage in hematopoietic development, as exemplified by the *AML-ETO* and *PML/RAR*α fusion gene that is associated with a block in differentiation. Thus, coexpression of a mutation that confers a proliferative and /or survival advantage and a mutation that impairs hematopoietic differentiation could result in acute leukemia.

The rapid diagnosis of the t(15;17)(q22;q21) in APL is important in the early introduction of targeted therapy with ATRA. Immunophenotyping is considered to be very useful for lineage assignment of immature and mature leukemic cells. Flow cytometry is the preferred method for immunophenotypic identification, enumeration and characterization of blast cells. The cytogenetics analysis of leukemic cells in metaphases is also an essential component of the diagnosis of t(15;17). The development of reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence *in situ* hybridization (FISH) technologies have supplemented metaphase cytogenetics. RT-PCR methods for the amplification of *PML/RAR*α transcripts have also been developed for the diagnosis and detection of minimal residual disease in APL in the western countries.

At present, no data exists in Thailand with respect to the incidence and type of *PML/RAR*α fusion gene in Thai APL patients. In this thesis, I set out to explore the frequency and characteristics of *PML/RAR*α fusion gene in our unique ethnic population and look for the specific immunophenotypic markers that could be used to identify and predict the presence of *PML/RAR*α fusion genes in APL patients. Moreover, I also searched for the additional genetic event that may coexist in APL patients with *PML/RAR*α gene, particularly *FLT3* mutation, which belongs to Class I mutation, in order to find evidence to support the model of multiple cooperative genetic events that could also occur in Thai APL patients. I hope that the results of

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this research would guide us how to diagnose and predict the presence of *PML/RAR*α fusion genes in our leukemia patients by flow cytometry. The development of molecular assays to detect the genetic abnormalities in leukemic cells would also allow us to better understand the leukemogenetic steps that are central to the pathogenesis of APL.

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CHAPTER II

OBJECTIVES

1. To determine the frequency and type of *PML/RARα* fusion gene *variants* (*bcr1, bcr2 or bcr3*) and their association with leukemic karyotype and clinical presentations of Thai AML patients.
2. To explore if specific immunophenotypic markers can be identified to predict the presence of *PML/RARα* fusion genes in AML patients.
3. To determine the additional genetic event, in particular, *FLT3* mutation, that may coexist in APL patients with *PML/RARα* fusion gene.

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CHAPTER III
LITERATURE REVIEW

1. Acute myeloid leukemia (AML)

1.1 Definition

Acute myeloid leukemia (AML) is a cancer of white blood cells, characterized by an increase in the number of myeloid cells in the marrow and an arrest in their maturation, frequently resulting in hematopoietic insufficiency (granulocytopenia, thrombocytopenia, or anemia), with or without leukocytosis.

The AML phenotype seems to be maintained by an increased proliferation of these blast cells, which is considered to result from the combination of two components: (i) the block of differentiation hindering progenitors to reach the postproliferative stage and to undergo programmed cell death subsequently and (ii) an increased capacity for self-renewal of the leukemic progenitors (1).

1.2 Signs and symptoms

Signs and symptoms of acute leukemia result from infiltration of bone marrow or extramedullary sites by blasts. As a result, initial symptoms may be due to the presence of anemia, neutropenia, or thrombocytopenia. Patients generally present with nonspecific complaints including weakness, lethargy, fatigue, dyspnea, fever, weight loss, or bleeding. Blasts may also infiltrate organs or lymph nodes, resulting in hepatosplenomegaly or adenopathy. Bone marrow infiltration with blasts can result in bone pain. Physical findings may include pallor, lymphadenopathy, hepatomegaly, splenomegaly, or bone tenderness. Mucosal bleeding, petechiae, ecchymosis, and fundal hemorrhages may occur as a result of thrombocytopenia.

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1.3 Types of acute myeloid leukemia (AML)

The original classification scheme proposed by the French-American-British (FAB) Cooperative Group divides AML into 8 subtypes (M0 to M7). Although AML blasts evolve from common myeloid precursors, the 8 subtypes differ in degree of maturation (Table1) (2, 3). The FAB classification of AML is based on morphology and cytochemical staining of blasts (4). However, the recent classification schemes proposed by the World Health Organization (WHO) require the additional evaluation of the leukemic blasts by molecular analysis and flow cytometry. Table 2 summarizes the new classification of AML as proposed by WHO (5-7).

In the subgroup "AML with recurrent genetic abnormalities," the WHO recognizes 4 well-defined recurring genetic abnormalities (Table 2) that are usually associated with de novo AML. Nearly 30% of patients with AML will have one of these genetic abnormalities. In cases of AML with t(15;17), t(8;21), and inv(16) or t(16;16), there is a strong correlation between the genetic findings and the morphology that the genetic abnormality can usually be predicted from the microscopic evaluation of the blood and marrow specimens.

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Table 1 French-American-British (FAB) classification of acute myelogenous leukemia**.**

FAB Type	**Descriptive Term**
M0	Acute myeloblastic leukemia, undifferentiated
M1	Acute myeloblastic leukemia, without maturation
M2	Acute myeloblastic leukemia, with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Erythroleukemia
M7	Acute megakaryoblastic leukemia

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Table 2 WHO classification of acute myeloid leukemia

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;q21), (*PML/RAR*α) 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

Classify 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

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1.4 Multi-step pathogenesis of AML

There are many data suggest that at least two mutations are required development of AML (Figure1) (8-10). The epidemiologic data indicate that two broad classes of mutations are required. One class of (class I) mutation confers proliferative and/or survival advantage to hematopoietic progenitors and are usually gain of function mutations that result in constitutive activation of signal transduction pathways or their downstream effectors, for example *BCR/ABL*, *TEL/PDGFβR*, the hematopoietic receptor tyrosine kinase *FLT3* and *RAS* mutations. When express alone results in chronic myeloid leukemia (CML) like disease characterized by leukocytosis with normal maturation and function of cells. Class II mutations, result in loss of function of transcription factors that are important for normal hematopoietic differentiation and include the *AML1/ETO*, *CBFβ/SMMHC*, *PML/RARα* and *NUP98/HOXA9* fusions as well as point mutations in hematopoietic transcription factors such as AML1 and C/EBPα. These mutations would also be predicted to impair subsequent apoptosis in cells that do not undergo terminal differentiation. When expressed alone, these mutations may confer a phenotype most like myelodysplastic syndrome (MDS). Regardless of the timing or order of acquisition of mutations, individuals who accrue both Class I and Class II mutations have a clinical phenotpe of AML characterized by a proliferative and/or survival advantage to cells and by impaired hematopoietic differentiation. The hypothesis has important clinical therapeutic implications, For example, targeting the proliferative and survival pathways with *FLT3* inhibitor or inhibitions of *RAS.* Agents that block in differentiation in APL, as all tran retinoic acid (ATRA) may have therapeutic benefit.

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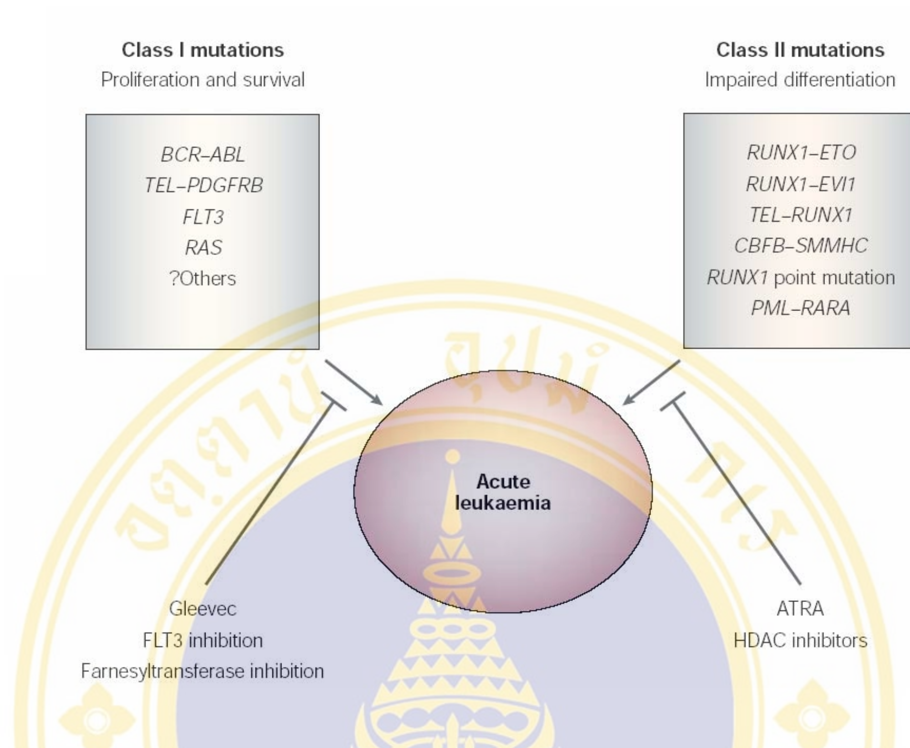

Figure 1 Multistep pathogenesis of AML and therapeutic implications. This model hypothesize that AML is consequence of cooperation between at least two broad classes mutation. The first class, exemplified by activating mutation in BCR/ABL, TEL/PDGFβR, FLT3 or RAS, confers a proliferative and/or survival advantage to hematopoietic progenitors, but not effect differentiation. When express alone results in CML- like phenotype. A second class of mutations, exemplified by loss of function mutations in hematopoietic transcription factors such as core binding factor or *PML/RARα*, result in impaired hematopoietic differentiation and may confer an immortalization phenotype due to the inability to undergo terminal differentiation and apoptosis. These mutations alone appear to confer a phenotype most similar to MDS like. 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. The hypothesis has important clinical therapeutic implications, For example, targeting the proliferaative and survival pathways with *FLT3* inhibitor or inhibitions of *RAS*. Agents that block in differentiation in APL, as all tran retinoic acid (ATRA) may have therapeutic benefit. (Nancy A. Spec and D. Gary Gilliland, 2002(3))

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1.5 Acute promyelocytic leukemia (APL)

1.5.1 Epidemiology of APL

Acute promyelocytic leukemia represents approximately 10-15% of AMLs in adults (10). The median age is approximately 40 years, which is considerably younger than the other subtypes of AML (70 years). There appears to be an increased incidence among Hispanic patients (20-30%). Finally, there is no apparent increase in incidence with age, unlike other subtypes of AML (11).

1.5.2 Morphological and cytochemical characteristics of APL

APL is an acute myeloid leukemia characterized by leukemic cells blocked at the promyelocytic stage of granulocytic differentiation. According to the French-American-Britsh (FAB) classification, two main cytological subtypes are recognized: (a) classical hypergranular promyelocytic leukemia (M3) and (b) hypogranular (microgranular) promyelocytic leukemia (M3v) (12).

Evaluation of the cell morphology and of the cytochemical reactions is usually sufficient to make a diagnosis of APL; nevertheless, careful examination can draw attention to certain additional details which may be of relevance to diagnosis Morphological analyses of APL were described as: (a) abnormal promyelocytes with a characteristic pattern of heavy granulations; (b) the nucleus varies greatly in shape and is often reniform or bilobed; (c) the cytoplasm of most of the cells is completely occupied by closely packed or even coalescent large granules.

1) Hypergranular promyelocytic leukemia

The leukemic cells are characterized by numerous coarse purple granules in the cytoplasm. The nuclear contour may be irregular, bilobed or folded. Sometimes the cells show a cytoplasm filled with heavy azurophil granules which totally obscure the nuclear outline. Multiple Auer bodies (Faggotts) are commonly found in some cells. Most (80%) patients with APL have characteristic hypergranular blasts.

2) Hypogranular (microgranular) promyelocytic leukemia variant (M3v)

Most of the leukemic cells are devoid of granules or contain only sparse fine granules. The abnormal promyelocytic cells, whether small or large, have bilobed

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or kidney shape nuclei and reveal a basophilic cytoplasm with staining characteristics ranging from faint to strong.

The cytochemistry of both forms of M3 shows characteristic strong positivity for peroxidase or Sudan Black B and for chloroacetate esterase. The bundles of Auer rods are recognized easily with the latter stains but not the former.

1.5.3 Clinical presentation of APL

APL is associated with a number of features that emphasize the need for rapid and accurate. Patients with APL will most often present with bruising and bleeding due to the hemorrhagic diathesis which is present in the majority of patients at diagnosis. In addition, patients present with fatigue due to anemia, or infection secondary to severe neutropenia. In a small number of cases, patients present with severe shortness of breath due to stasis of leukemic cells in the pulmonary vasculature. Because of the bleeding risk, APL should be viewed as a medical emergency.

1.5.4 Immunophenotypic features of acute leukemias

Modern immunologic classifications of leukemia reflect the development of monoclonal antibodies. Several hundred monoclonal antibodies have been assigned to Clusters of Differentiation (CD) groupings by the International Workshops on Leukocyte Differentiation Antigens. A variety of fluorochromes such as phycoerythrin (PE), peridinin chlorophyll protein (PerCP), conjugates are now available to complement the standard repertoire of fluorescein isothiocyanate (FITC). Modern multiparameter flow cytometers make full use of the newer fluorochromes, facilitating simultaneous studies of several different cellular antigens (13).

Leukocyte antigens are rarely lineage-specific. However, panels of monoclonal antibodies to relatively lineage-restricted antigens can be used to identify the lineage of leukemic cells in the vast majority of cases. Additional antibodies can then be selected on the basis of the results to identify stage of differentiation, prognostic features, or aberrant phenotypes for monitoring residual disease.

The immunophenotypic analysis of acute leukemia by flow cytometry has become a powerful tool for proper identification of myeloid or lymphoid lineage (14). Table 3 and Table 4 summarize panels that are clinically useful for

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immunophenotyping acute leukemias (15, 16). The immunophenotypic patterns of acute leukemias are well known. Immunophenotypic characterization of blast cells has several goals:

1) Lineage assignment

2) Evaluation of cell maturation

3) Assessment of phenotypic aberrations

Additional progenitor cell markers such as CD34 are used to confirm the immaturity of the pathologic cells. From the practical point of view, lineage assignment should typically be based on the combined use of markers for immaturity and for the different lymphoid (B, T) and myeloid lineages together with markers that allow the identification of blast cells.

i) Acute myeloid leukemia (AML)

The term 'acute myeloid leukemia' is often used to designate any of the nonlymphoid leukemias, including myelocytic (M0, M1, M2, M3), monocytic (M4, M5), erythroid (M6), and megakaryocytic (M7) subtypes.

APL has a characteristic immunophenotype with the majority of leukemic cells expressing the differentiation proteins MPO, CD13 and 33. Importantly, the cells have low to absent expression of CD34 and HLA DR (17-19). Class II HLA antigens (HLA DR), which are found on all hematopoietic precursors, are usually not detected or expressed on the surface of the malignant progranulocyte. The explanation and biologic implications of this finding are not known. Cells with this phenotype, but without the characteristic morphology of APL, should be suspected as being of the M3v type of APL.

ii) Acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemias (ALL) were the first group of hematological malignancies in which immunophenotyping proved to be clinically useful. More than 20 years ago, ALL was already classified as B and T ALL depending on whether leukemic cells expressed surface antigens.

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Table 3 Immunophenotypic patterns of AML patients.

Antigen	M0	M1	M2	M3	M4	M5	M6	M7
MPO	-	+	+	+	-/+	-/+	-/+	-/+
CD13	+/-	+	+	+	+	+	+	+
CD33	+/-	+	+	+	+	+	+	+
CD34	>80	>80	<80	-/+	-/+	-/+	-/+	-/+
CD14	-	-	-	-	<50	>50	-	-
CD41	-	-	-	-	-	-	-	+
GlyA	-	-	-	-	-	-	+	-
HLA-DR	+	+	+	-	+	+	+	+
CD19	-	-	-/+	-	-	-	-	-

Table 4 Immunophenotypic patterns of ALL patients.

Antigen	B precursor ALL	Pre- B ALL	B ALL	Pre-T ALL	T-ALL
CD3	-	-	-	+	+
CD5	-	-	-	+	+
CD7	-	-	-	+	+
CD10	+	+	+/-	-	-
CD19	+	+	+	-	-
CD20	+/-	+/-	+	-	-
CD22	-	-	+	-	-
CD34	+	+/-	-	+	-
TdT	+	+/-	-	+	-
Cytoplasmic µ	-	+	-	-	-
Surface Ig	-	-	+	-	-
HLA-DR	+	+	+	+	+

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1.5.5 Molecular genetics of acute promyelocytic leukemia

APL represents a uniquely homogeneous subset of AML defined by its cytogenetic abnormality, t(15;17), which results in fusion of the retinoic acid receptor (RAR) α gene on chromosome 17 with the promyelocytic leukemia (PML) gene on chromosome 15 (20-22). This abnormality yields the PML/RARα fusion protein, detectable by PCR techniques, which is useful for both diagnosis and evaluation of minimal residual disease.

1.5.6 In vitro cell lines and animal model of APL

1) Cell lines

Four cell lines have been reported to be derived from patients with APL: HL-60, and NB-4. HL-60 lack t(15;17) while NB-4 carries this cytogenetic hallmark for APL.

i) HL-60

In 1980, the HL-60 myeloid cell was considered to be derived from promyelocytic leukemic cells (23). However, we now know that this cell is not a promyelocytic leukemic cell because it does not carry the specific 15;17 translocation and it only possesses one chromosome 17 (24).

ii) NB-4

The first, genuine human promyelocytic cell line with t(15;17) has recently been established from the bone marrow of a patient with morphologically, cytogenetically and clinically typical APL. NB-4 cells were instrumental in the molecular characterization of the L (long) isoform *PML/RAR*α fusion gene (25).

iii) UF-1

UF-1 APL cell line developed from a patient clinically resistant to all-trans RA. Cell surface markers in the UF-1 cells were positive for CD7, CD13, CD33, and CD38. Cytogenetic analyses revealed additional abnormalities, 46XX. add(ll(q44). add(6)(q12), add(7)(q36), t(15;17) (q21;q21)(23). Molecular analyses showed a S (short) isoform (26) *PML/RAR*α fusion transcript. This RA-resistant APL cell line may be a useful model for molecular studies on the block of leukemic cell differentiation and as a means to investigate the mechanisms of RA resistance.

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iv) HT93

HT93 cell line established an APL. HT93 has the capacity to differentiate into neutrophils and eosinophils in response to all-trans retinoic acid (ATRA) and human hematopoietic cytokines. The cells had a myeloblastic morphology, were positive for surface CD33, CD34, and CD56, and showed the following karyotypes: 46, XY, t(1;12)(q25;p13), 2q+, t(4;6)(q12;q13), and t(15;17)(q22;q11). When the cells were cultured with ATRA, they showed nuclear segmentation and developed secondary granules consisting in part of neutrophils and eosinophils. HT93 is an APL cell line with the ability to differentiate into neutrophils and eosinophils. With RT-PCR, a short type *PML-RAR*α transcript was detected (27).

2) Animal models of APL

Several laboratories have tried to model APL in animals (Table 5). The first transgenic mouse created that expressed the *PML/RAR*α fusion used the CD11b promoter, which was expressed relatively late in myeloid maturation. These mice did not develop APL or a preleukemic syndrome.

Two groups performed transgenic experiments with *PML/RAR*α using the cathepsin G promoter. These mice developed a preleukemic syndrome characterized by an increase in immature myeloid forms in the bone marrow and splenomegaly due to extramedullary hematopoiesis. About 10% to 30% of the animals developed leukemia, with a median latency of 300 days.

The level of *PML/RAR*α expression must be finely monitored a comparison of cathepsin-G-driven *PML/RAR*α (28) expression in transgenic versus knock-in mice, has shown that lower levels of *PML/RAR*α expression observed in the knock-in mice resulted in increased penetrance of the disease. These mice first show a progressive myeloproliferation, later followed by the promyelocyte-specific differentiation block and acute leukemias with promyelocyte features. Depending on the promoter used, the level of transgene expression and the strain background, APL develop in 6 to 18 months, indicating that as yet undefined secondary genetic changes are required for full APL development.

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Table 5 Transgenic mouse models of acute promyelocytic leukemia.

Transgene	**Promoter used to regulate expression**	**Phenotype**	**Cell type targeted for transgene expression**	**References**
PML/RARα (L)	Cathepsin G	APL	Promyelocyte	(30-32)
PLZF/RARα	Cathepsin G	Myeloid leukaemia with maturation*	Promyelocyte	(33)
NUMA/RARα	Cathepsin G	Myeloid leukaemia with maturation*	Promyelocyte	(33)
PML/RARα (L)	MRP8	APL	Promyelocyte, basal keratinocyte	(34)
PML/RARα-R4	MRP8	Retinoic acid resistant APL	Promyelocyte, basal keratinocyte	(35)
PML/RARα-K160R	MRP8	Myeloproliferation	Promyelocyte, basal keratinocyte	(36)
PML/RARα (S)	CD11b	Defects in haematopoiesis	Granulocytes	(37)
PML/RARα (L)	Metallothionein	Normal haematopoiesis, liver disease	Ubiquitous expression	(37)

*These cells do not undergo a block in differentiation, so differentiated cells of leukaemic origin can be found in the blood or bone marrow. APL, acute promyelocytic leukemia; MRP8, macrophage-related protein 8; *NUMA/RARα*, nuclear mitotic apparatus retinoic acid receptor-α fusion protein; *PML/RARα*, promyelocytic leukemia retinoic acid receptor-α fusion protein; *PML/RARα* (L), large form of the *PML/RARα* fusion protein, associated with better prognosis; *PML/RARα* (S), small form of the *PML/RARα* fusion protein, associated with worse prognosis; *PML/RARα*-R4 contains a mutation that blocks retinoic acid-binding.

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The mouse model of APL most similar to human disease was generated by use of the MRP8 promoter, which is expressed at the promyelocyte to metamyelocyte stage and continues to be active in mature neutrophils (29). These mice also developed a preleukemic phase, and about one third developed promyelocytic leukemia with a median latency of 6 months.

In mice, expression of *X/RAR*α fusion proteins associated with human APL results in myeloid leukemias in which cells have fusion protein (the reciprocal of the *PML/RAR*α fusion protein) increases the penetrance of APL in mice. Although this reciprocal fusion transcript can be detected in 60% of patients with t(15;17) associated APL, none of the proteins encoded by *RAR*α*/PML* have been detected in patients, so far. Nevertheless, studies in mice indicate that the reciprocal fusion proteins could modulate the phenotype of *X/RAR*α-transformed cells.

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2. Promyelocytic leukemia (PML)

2.1 Nuclear structure

The nuclear matrix consists of two parts, the nuclear lamina and an internal fibrogranular network made of protein and RNA. This internal matrix is a network of irregular fibers that have an intricate fine structure. Structural remnants of nucleoli are evident in this network. The fibers are built on a network of branched filaments that lie beneath and protrude from these fibers. The nuclear matrix binds diverse nuclear proteins and supports their assembly into functional macromolecular complexes involved in important nuclear processes, such as transcription, RNA splicing and DNA replication.

Normal nucleus (Figure 2A), the nucleus is bounded by the nuclear lamina, a proteinaceous layer made of the lamins and associated proteins. The lamina is connected on its cytoplasmic face to the double-membrane nuclear envelope. On its inner surface, the lamina binds to chromatin and in most cell types the lamina associated chromatin domains correspond to heterochromatin. Another key site of heterochromatin formation is at the surface of the nucleoli. Typically, 1-3 nucleoli are present per nucleus. These have a well-established role in ribosome biogenesis, but seem to also be involved in other functions such as mRNA transport, p53 metabolism and the control of proliferation. The nuclear matrix (nuclear matrix proteins (NMP)) is the non-chromatin nuclear scaffolding that participates in the spatial organization of chromatin and the positioning of nuclear molecules and substructures. One such substructure is the promyelocytic leukemia (PML) body. The PML growth and tumor suppressor is an essential component of this doughnut-shaped multiprotein complex. One important function of PML and the PML body is the control of various apoptotic pathways.

In tumor cells (Figure 2B) are examples of nuclear changes. Nuclei can become irregular and begin to fold, and coarse heterochromatin aggregates are frequently observed. Nucleoli can be enlarged and PML bodies can mislocalize in microspeckles.

Furthermore, appearance of the perinucleolar compartment is observed in tumor cells, and specific NMPs might be absent. It is important to note that not all of

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these changes are observed simultaneously in nuclei of actual cancer cells different abnormalities are associated with different cancer types.

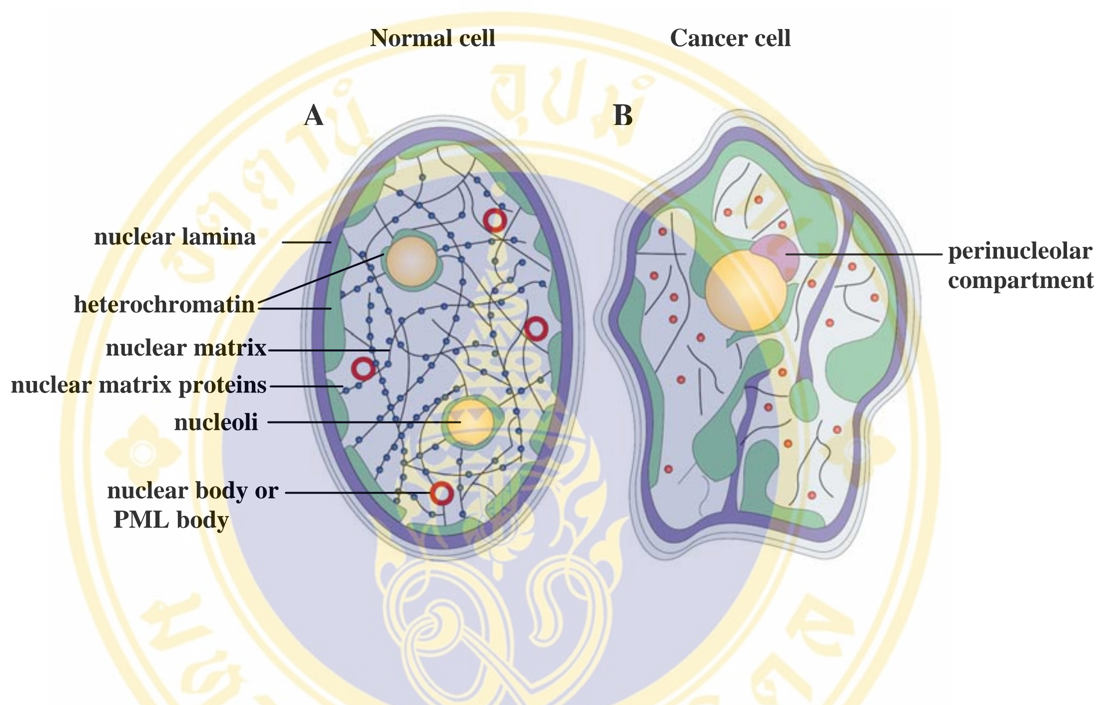

Figure 2 Nuclear structure in normal (A) and cancer cells (B). Normal nucleus, the nucleus is bounded by the nuclear lamina. The lamina is connected on its cytoplasmic face to the double-membrane nuclear envelope, the lamina binds to chromatin and in most cell types the lamina associated chromatin domains correspond to heterochromatin. Typically, 1-3 nucleoli are present per nucleus. The nuclear matrix participates in the spatial organization of chromatin and the positioning of nuclear molecules and substructures. The promyelocytic leukemia (PML) body is substructure. The PML growth and tumor suppressor is an essential component of this doughnut-shaped multiprotein complex, the function of PML and the PML body is the control of various apoptotic pathways. In tumor cells, nuclei can become irregular and begin to fold, and coarse heterochromatin aggregates are frequently observed. Nucleoli can be enlarged and PML bodies can mislocalize in microspeckles. (Zink *et al,* 2004 (38))

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PML nuclear bodies

PML is expressed in the nuclear diffuse fraction of the nucleoplasm as well as in a discrete sub-nuclear compartment, the NB. This structure is also known by a variety of names, including ND10 (nuclear domain 10), Kremer (Kr) bodies, PODs (PML oncogenic domains) and PML bodies (39), that is altered in cancer cells. Normal cells contain about 10-30 PML bodies in their nuclei and these doughnut shaped multiprotein complexes have diameters of approximately 0.2-1.0 μm although their number and size change during the cell cycle. They contain the tumor suppressor PML, which is essential for PML body integrity, and more than 50 other proteins are known to assemble, either transiently or constitutively, within PML bodies. Several studies indicate that PML bodies are also involved in DNA repair and genomic stability, as well as in growth suppression and cellular senescence in response to oncogenic transformation and DNA damage .The role of PML and the PML body has been most extensively studied in patients with APL, which is associated with certain chromosome translocations most often between the *PML* gene on chromosome 15 and the *RAR* α gene on chromosome 17.

2.2 PML structure and functions

PML contains several well characterized motifs (Figure 3) (28, 40), and a great deal of research has focused on how these domains contribute to the multiple biological functions that have been attributed to the protein. The PML locus is approximately 35 kb in length and contains nine exons.

Alternative splicing of C-terminal exons yields up to 20 different isoforms of the protein. Most cell lines express a similar pattern of isoforms, and the exact functions of these isoforms are not clear. All PML isoforms share the N terminal amino acid region, but differ in their central and C terminal portions. C-terminal sequences for specific PML isoforms play a role in regulation of PML stability and interaction with the p53 tumor suppressor protein (39). The PML gene is ubiquitously expressed and encodes a nuclear protein characterized by a modular structure which includes a set of zinc finger domains known as the RING and B-boxes and a-helical coiled-coil motif responsible for mediating interaction with multiple proteins, nuclear localization, PML multimerization, and heterodimerization with *PML/RARα*.

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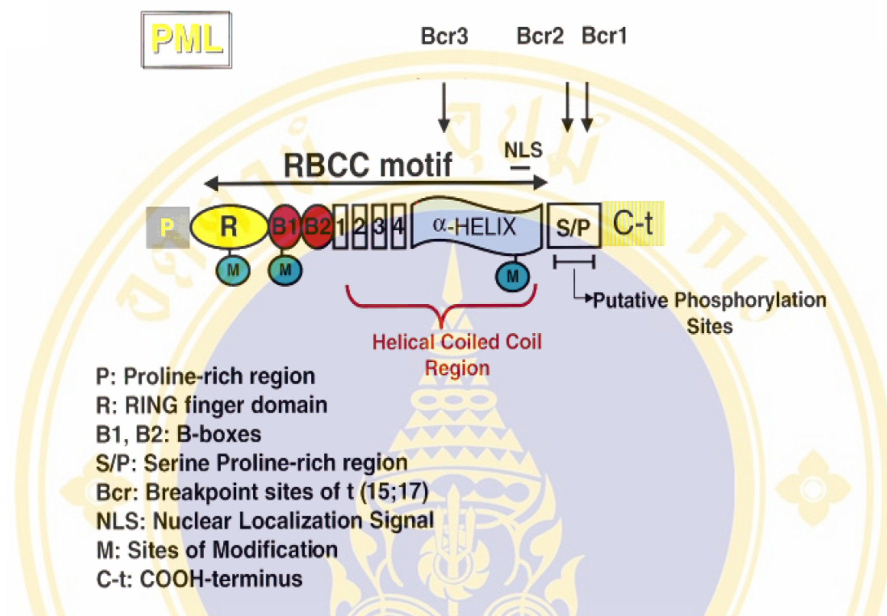

Figure 3 Schematic representation of the modular organization of the PML protein. The 1, 2, 3, 4 clusters consist of hydrophobic amino acids heptads which form a part of the domain known as the helical coiled-coil region. Collectively the RING finger domain (R), the B-boxes (B1 and B2), and the helical coiled coil (CC) region form the RBCC motif. The RBCC motif is shared by a growing family of proteins. This region has been implicated in mediating homodimerization, the localization of PML within the NB, interaction with other proteins including *PML/RARα*, as well as PML growth suppressive abilities. The carboxyl-terminus of PML is variable depending on alternative splicing of the PML 3' exons. The position where PML fuses to RARα as a consequence of the three distinct break point cluster regions within the PML locus are indicated by the arrows (Ruggero *et al*, 2000 (41)).

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The PML protein contains several important functional domains which collectively form the RBCC motif. This is characterized by the presence of a zinc-binding domain that includes the RING finger motif followed by two cysteine/ histidine-rich B-boxes (B1 and B2), and a-helical coiled coil domain. The Ring finger domain is involved in mediating interactions between proteins and is present in several proteins associated with cellular transformations, including the human tumor suppressor, in which an identified cancer predisposing mutation results in the deletion of the Ring finger. Binding of zinc ions to the cysteine/ histidine-rich protein motif called the B-box (B1 and B2) induces changes in the structure of the peptides. The helical coiled-coil region consists of eight heptads repeats and is responsible for multimerization of PML as well as its heterodimerization with *PML/RAR*α. In addition to the RBCC motif, a nuclear localization signal (NLS) is present within the PML protein, and its deletion results in a cytoplasmatic and perinuclear PML localization pattern.

The RBCC motif is important for the localization of PML within the nuclear bodies as well as its growth suppressive abilities. Individual deletions in any one of the domains that compose the RBCC motif have no effect on PML growth suppressor activity, suggesting that there is no single component of the RBCC motif that is indispensable for the growth inhibitory effects of PML. The RBCC domain of PML is highly conserved and is shared by a growing family of proteins.

The precise physiological functions and biochemical activities of PML are not entirely clear. However, PML has been implicated in a number of diverse cellular processes, including apoptosis, cellular senescence, growth regulation, transcriptional regulation, RNA-processing, control of genomic stability, response to viral pathogens, antigen presentation and tumor suppression

1) Cellular Proliferation

The expression of PML and other PML-NB components is markedly induced by type I and II interferon. As a consequence, the number and size of PML-NBs per nucleus increases. This suggested that PML and the PML-NB could mediate IFN-regulated cellular functions, including growth and tumor suppressive activity. Indeed, in over expression studies, in tumor cell lines from various histological origins, PML acts as a potent growth suppressor by inducing a block in the G1 phase of cell cycle.

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2) Growth and tumor suppression of PML

In over expression studies, PML acts as a potent growth suppressor in several cell lines of various histological origins. The ability of PML to regulate the cell cycle may be, in part, due to the ability of PML to interact with retinoblastoma protein (pRB). PML retains a growth suppressive activity in Rb deficient cells, suggesting that PML is likely to affect multiple pathways involved in cell-cycle regulation. In vitro experiments show that PML can act as a tumor suppressor.

3) PML and control of apoptosis

PML is important for the induction of multiple caspase dependent apoptotic signals including Fas, tumor necrosis factor (TNF), ceramide and IFN mediated apoptosis. In addition, the overexpression of PML in various cell lines results in increased programmed cell death. It has also been reported that overexpression of PML induces apoptosis in the absence of caspase activation. However, the mechanisms by which PML acts as a proapoptotic factor remain largely unknown. Since PML can act as a transcription co-factor, it is possible that PML might regulate the expression of genes involved in apoptotic pathways.

4) Transcriptional regulation

An accumulating body of evidence demonstrates that PML regulates the expression of target genes at the transcription level. This could explain, at least in part, how PML exerts its biological functions. PML has been shown to both repress and activate transcription. However, PML can also act as a transcriptional coactivator.

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2.3 PML action in APL

The PML nuclear body is a target of the *PML/RARα* oncoproteins in APL. In APL the *PML* gene is translocated and fuses to the *RAR*α gene, leading to the formation of fusion genes that encode *PML/RARα* and *RARα/PML* fusion proteins. *PML/RARα* can interfere with the transcription function of RARα, thus impairing myeloid differentiation. The fusion protein can also form heterodimeric complexes with PML which delocalize PML from the NB resulting in the disruption of this structure. *PML/RARα* can, therefore, interfere with PML function, as well as the function of other NB components. In view of the critical role of PML in growth and tumor suppression, interference with PML function by the action of *PML/RARα* might result in a growth advantage of leukemic blasts. In fact, in transgenic mice, *PML/RARα* renders hemopoietic progenitor cells resistant to FAS-, TNF- and IFN-induced apoptosis.

The *PML/RARα* fusion gene product always contains the regions of RARα (B-F), including the nuclear receptor DNA and ligand-binding domains. The N-terminal PML sequences show patient to patient variability determined by the position of the translocation breakpoint within the PML gene and by alternative exon splicing. Pandolfi, et.al (42), described three breakpoint clusters within the PML gene located in intron 3 (bcr3 or short form), exon 6 (bcr2 or variable form) and intron 6 (bcr1 or long form).

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3. Retinoic acid receptor alpha (RARα)

3.1 Retinoids

3.1.1 What is a Retinoic acid?

Retinoic acids (RAs) are signaling molecules that, together with their nuclear retinoid (RARα, β and γ) and rexinoid (RXRα, β and γ) receptors (43, 44), establish genetic communication networks that are essential for embryonic development (43). But they also have important physiological functions, particularly in the brain and reproductive system, by regulating organogenesis, organ homeostasis, and cell growth, differentiation and death.

3.1.2 Origin of retinoids

Retinoids are formed from dietary vitamin A, particularly from eggs, milk, butter and fish-liver oils, and the provitamin β-carotene of plant origin. After uptake by the intestinal mucosa cells, retinol (vitamin A) is esterified to retinyl esters before passing into the lymphatics and being transported through the blood in chylomycrons, from where they are taken up by the liver and stored. After mobilization by cleavage of retinyl esters, retinol is oxidized to retinal and retinoic acid (RA). Gene-ablation experiments have shown that, for the latter step, retinaldehyde dehydrogenase-2 is the key enzyme in the post-implantation mammalian embryo; its absence leads to early embryonic death.

A very small proportion of plasma and tissue retinol (0.2–5%) is converted to all *trans* RA (ATRA), the main activator of RA receptors (RARs) but not rexinoid receptors (RXRs). Other metabolites with signalling activity include 9-*cis* RA and 13-*cis* RA (most commercially available preparations of ATRA contain at least 1% 9-*cis* RA as a contaminant). 13-*cis* RA is a metabolite of ATRA that has weaker activity than all-*trans* and 9-*cis* RA in transcription assays. The precise origin of 9-*cis* RA,which binds to and activates transcription from RARs and RXRs, is less clear. It is still not proven that 9-*cis* RA acts as a physiological ligand of RXRs. Other endogenous ligands have also been described. The occurrence and physiological significance of other retinoids, such as the 4-oxo- and didehydro- derivatives, and 1,4-hydroxy-4,14-*retro*retinol, are largely unknown.

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3.1.3 Mechanism of retinoid action

RAs do not act solely through the two subunits of the RAR-RXR heterodimer. RARs and RXRs activate or repress gene transcription by recruiting multiprotein co-activator and/or co-repressor complexes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity.

RXR is a promiscuous heterodimerization partner for various NRs (nuclear receptor). In the absence of ligands for RAR-RXR dimers, or in the presence of some antagonists, the receptors target genes are repressed. This is due to the recruitment of histone deacetylase (HDAC)-containing complexes that are tethered through corepresors (CoRs) such as nuclear receptor (NR) corepressor (NCoR) or silencing mediator for retinoid and thyroid hormone receptors (SMRT) to the nonliganded RAR-RXR dimer. This process results in histone deacetylation, chromatin compaction and silencing of target gene promoter regions. Histone acetyltransferase (HAT) complexes, recruited by p160 CoAs lead to chromatin decondensation over the target gene promoter. Consequently, transcription is activated by two mechanisms: derepression, caused by chromatin decondensation, and a receptor dependent increase in the frequency of transcription initiation. HATs can also acetylate proteins other than histones, such as p53, but the functional link with NR action, if it exists, is unclear. RXR is a promiscuous heterodimerization partner for various NRs. Indeed, it has been shown that rexinoid agonists can induce differentiation programmes in the presence of elevated cyclic AMP levels independently of RAR signalling, and that they can autonomously induce rapid apoptosis under certain conditions.

Retinoic acid is essential for life

RA is an essential regulator of embryogenesis, and its production needs to be tightly controlled for proper organogenesis. In adults, retinoids and/or their cognate receptors are required for the proper functioning of a number of organs, including the skin, lung, liver, and neuronal and immune systems (45).

Antitumor activity of retinoic acids

Many factors can contribute to tumorigenesis, including inherited and acquired genetic changes, chromosomal rearrangements, epigenetic phenomena and chemical carcinogenesis. RAs can interfere with these events at several levels, their principal

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known actions being induction of differentiation and/or apoptosis of tumor cells, and inhibition of tumor promotion in chemically induced cancers. In keeping with their ability to regulate growth and induce differentiation throughout life, retinoid affect the growth of many tumor cell lines in culture.

3.2 Structure and function of RARα

RARα is a protein with a modular structure similar to that of other nuclear receptors. It consists of six evolutionarily conserved domains, designated A through F (28, 40) (Figure 4). RARα binds to RAREs through the C domain, which is the most conserved domain among the nuclear receptor superfamily. Two other domains, AF1 (A/B domains) and AF2 (E domain), co-operate to activate transcription. AF1, contained within the N-terminal A/B domain, is a ligand-independent transcriptional activation domain, which works in a promoter context-dependent manner. The AF2 domain is a ligand-dependent transcriptional activation domain as well as a dimerization interface for RXR. This domain associates with co-repressor or co-activator molecules in the absence or presence of ligand, respectively.

RARα is a ligand-dependent transcription factor stimulated by ATRA while its partner, RXR, responds to ATRA or 9-cis retinoic acid. These receptors bind as RAR/RXR heterodimers or as RXR homodimers to DNA motifs known as RA response elements (RAREs), thereby activating transcription of RA target genes. In addition, RXRs serve as partners for other nuclear receptors such as those for thyroid hormones and vitamin D3 integrating different signalling pathways.

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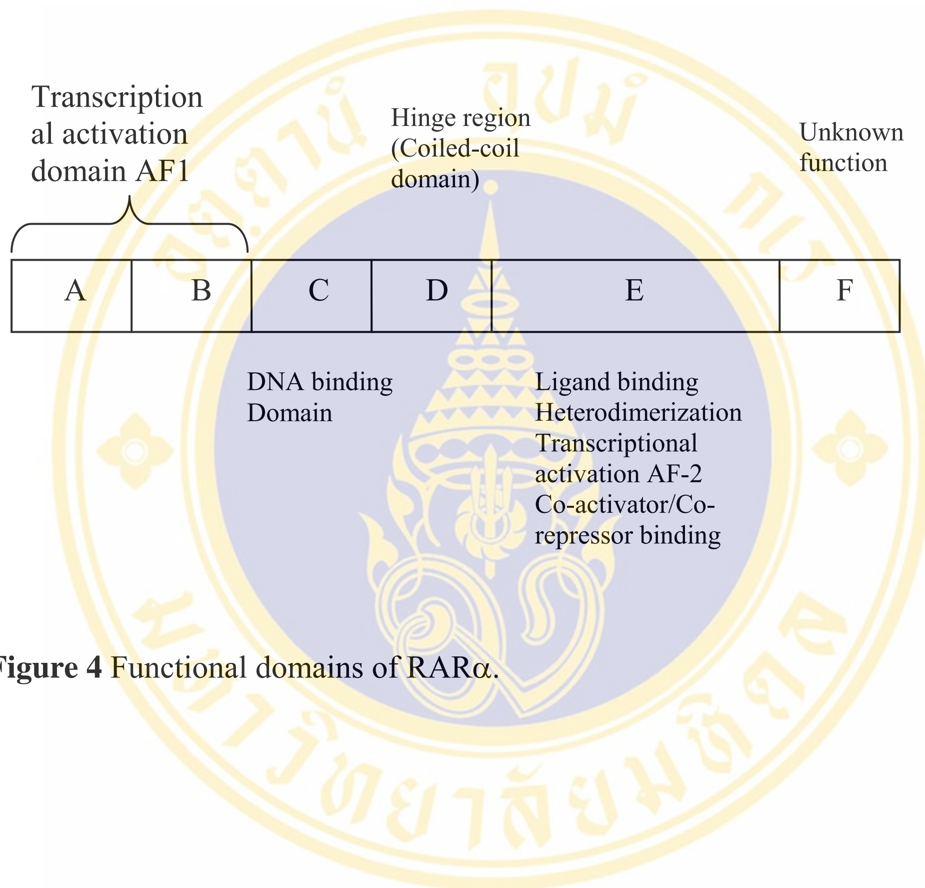

Figure 4 Functional domains of RARα.

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Transcriptional function of RARα

RARα, one of the three retinoic acid receptors, is a transcription factor of the nuclear receptor superfamily, which binds DNA when bound to another nuclear receptor, RXR (retinoid X receptor). In the absence of RA, RARα is a repressor of transcription; the RAR/RXR heterodimer recruits a repression complex containing nuclear receptor corepressors SMRT or N-CoR, corepressors mSin3, and histone deacetylases (HDACs). Deacetylation of core histones results in chromatin condensation and transcriptional repression. RA induces the dissociation of this complex and promotes the association of a coactivator complex containing nuclear receptor coactivator p160 family members and histone acetyltransferases CBP/p300. Acetylation of core histones leads to chromatin relaxation, promoter clearance, and activation of gene transcription, whereas in the presence of retinoic acid, it becomes a potent activator of transcription.

RARα and myeloid differentiation

Although other retinoic acid receptors have key roles in embryogenesis, RARα finely tunes the differentiation of granulocytes through the control of unidentified target genes.

Several lines of research underscore the importance of RARα in normal myeloid differentiation. RARα is expressed preferentially in myeloid cells, and retinoids preferentially stimulate granulopoiesis.

3.3 RARα action in APL

All reported cases of APL involve the fusion of RAR*α* to one of five different partners (designated X), resulting in the production of the fusion proteins *X/RARα* and *RARα/X*. The *X/RARα* fusion protein is always expressed and is consistently implicated in the development of APL. On the other hand, the *RARα/X* fusion protein may or may not be detected.

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4. *PML/RARα*

4.1 *PML/RARα* and hematopoietic stem cells (HSCs)

Neoplastic cells and normal stem cells share the capacity of unlimited self-renewal. Leukemias are considered to be malignancies initiated by genetic alterations occurring in HSCs (46, 47). Following the cell division of a normal hematopoietic cell, the 'daughters' of the 'stem cell' can either be committed to differentiate, to undergo apoptosis, or to remain a stem cell with the potential for self-renewal. These cell fate 'decisions' determine the composition of the cell pool constituting normal hematopoiesis.

PML/RARα is immediately present in the cell in which the t(15;17) occurs. The occurrence of t(15;17) already at the early stem cell levels may influence not only the functionality of myeloid precursors in the bone marrow but also of other cells derived from the early stem cell. The induction of *PML/RARα* expression at the promyelocytic stage of differentiation would correspond to a late appearance of the t(15;17) translocation. At the promyelocytic stage of differentiation, the cells should be fully committed to the granulocytic/monocytic differentiation, most likely without an influence on the erythroid and megakaryocytic differentiation pathways.

4.2 The multiple oncogenetic function of *PML/RARα*

PML/RARα can affect both PML and *RARα* functions (48). A detailed analysis of the biological activity of *PML/RARα* soon rendered obvious that this molecule exerted, both *in vivo* in transgenic mice and *in vitro* in hemopoietic cell lines, multiple oncogenic functions such as the ability: (i) to block myeloid differentiation; (ii) to confer a survival advantage and possibly a proliferative advantage to the expressing cell; and (iii) to render the cells genomically unstable. How *PML/RARα* could exert so many diverse oncogenic activities at the molecular level remains a puzzling question.

4.2.1 *PML/RARα* blocks differentiation of myeloid hematopoietic progenitors

This notion is supported by *in vitro* experiments in leukemic cell lines such as U937. These cells would normally differentiate in response to RA. The RA

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differentiating activity in U937 is abrogated as a consequence of *PML/RAR*α expression. Transgenic mice develop an APL-like leukemia *in vivo*, these leukemias are preceded by a long pre-leukemic phase characterized by a block of differentiation at the promyelocytic stage (48) that results in the slow and progressive accumulation of promyelocytes in the bone marrow and spleen of these transgenic mice.

4.2.2 Role of *PML/RAR*$\boldsymbol{\alpha}$ in control of cellular proliferation and survival

The fusion protein of APL can confer a proliferative and survival advantage to the myeloid hemopoietic cells. It can also favor the accumulation of additional genetic lesions rendering the cells genomically unstable.

In *vivo*, *PML/RAR*α causes leukemia only when expressed in the myeloid promyelocytic compartment. *PML/RAR*α in transgenic mice results in embryonic lethality. While it is still unclear why *PML/RAR*α can lead to oncogenic transfomation only in cells of specific histological origins, the net result of *PML/RAR*α activity cannot rely solely on its ability to affect myeloid differentiation. In transgenic mice, the phase that precedes leukemia onset is characterized by a progressive accumulation of cells with promyelocytic features in the spleen and the bone marrow; this expansion can be due to an increased proliferative rate and/or to a survival advantage. While the leukemic cells from *PML/RAR*α transgenic mice are found in active proliferation and can form an increased number of colonies in *in vitro* bone marrow cultures.

PML is essential for the induction of apoptosis and growth arrest upon multiple cellular stresses. PML mediates both p53-dependent and p53-independent apoptotic responses. *PML/RAR*α can block these PML pro-apoptotic activities. Although a through cell cycle analysis of hemopoietic cells harboring *PML/RAR*α has not yet been performed, it is logical to propose that the fusion oncoprotein could also lend a proliferative advantage to the APL blasts through its ability to impair the PML function.

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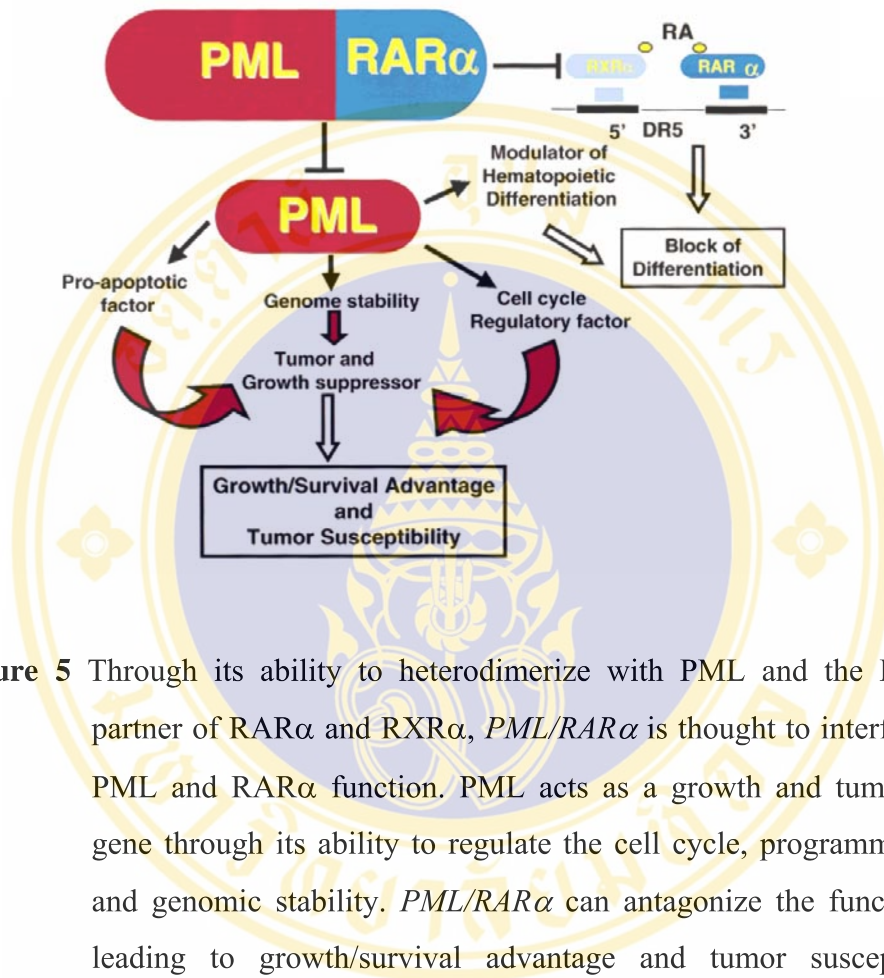

Figure 5 Through its ability to heterodimerize with PML and the DNA binding partner of RARα and RXRα, *PML/RARα* is thought to interfere with both PML and RARα function. PML acts as a growth and tumor suppressor gene through its ability to regulate the cell cycle, programmed cell death and genomic stability. *PML/RARα* can antagonize the function of PML, leading to growth/survival advantage and tumor susceptibility. The RA/RAR/RXR pathway has been implicated in controlling myelopoiesis. PML has also been shown to play a role in regulating the homeostasis of the myeloid compartment and mediating the RA response of these cells. Therefore, the concomitant dominant interference of *PML/RARα* with both PML and RARα pathways may lead to a block at the promyelocytic stage of hemopoiesis which characterizes APL (Ruggero D. et al., 2000 (41)).

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4.3 *PML/RARα* variants

Among APL cases with a *PML/RARα* rearrangement, chromosome 15 breakpoints fall within three breakpoint cluster regions (bcrs) that have been completely sequenced, although occasional patients have breakpoints outside these regions. Bcr1 and bcr3 correspond to *PML* introns 6 and 3 or 4, respectively, whereas bcr2 is located within *PML* exon 6 (Figure 6). Bcr1 breakpoints result in an mRNA fusion of *PML* exon 6 to *RARα* exon 3, conventionally referred to as the L (long) isoform. Bcr2 breakpoints usually result in a tripartite mRNA fusion in which part of *PML* exon 6 is fused to an insert sequence and then to *RARα* exon 3 referred to as the V (variable) isoform. Bcr3 breakpoints result in an mRNA fusion of *PML* exon 3 (bcr3a) (49, 50) or exon 4 (bcr3b) (51) to *RARα* exon 3, referred to as the S (short) isoform (Figure 7). The mechanism by which the t(15;17) translocation occurs and what determines the breakpoints is unknown.

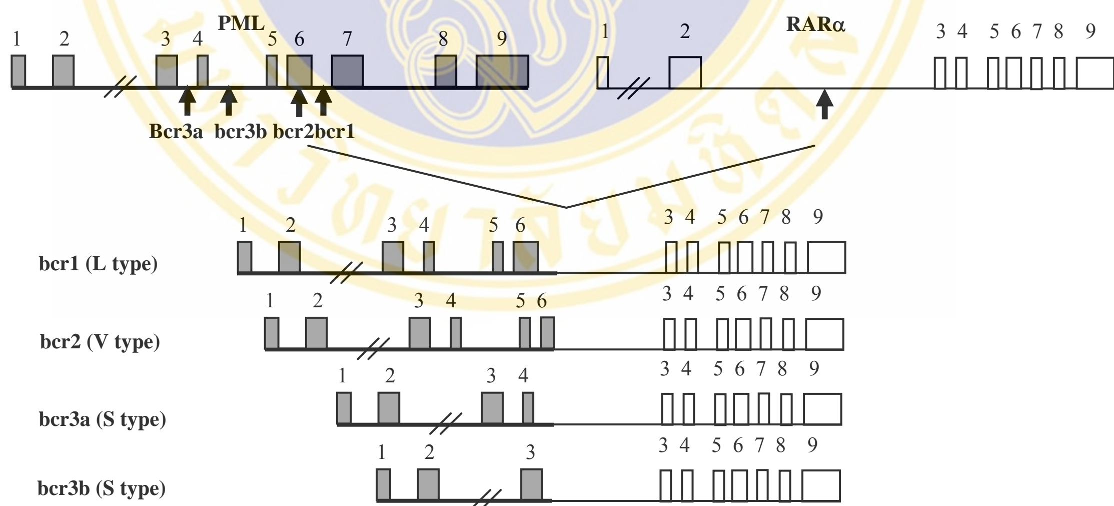

Figure 6 Diagram of PML and RARα loci. The breakpoint regions are indicated by arrows.

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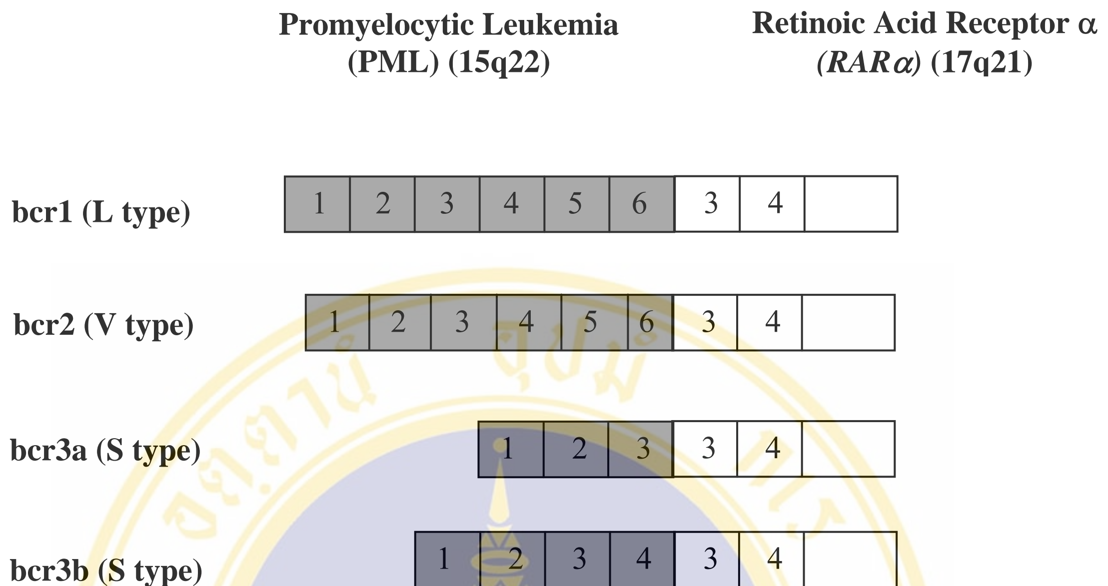

Figure 7 Schematic representations of the three major *PML/RARα* fusion transcripts. Breakpoints on the RARα gene always occur within intron 2. Due to distinct breakpoints on the PML gene (intron 6, exon 6, intron 3 and intron 4), different segments of this latter are fused to RARα exon 3, resulting in bcr1 (L type), bcr2 (V type), bcr3a (S type) and bcr3b (S type) fusion transcripts, respectively. Numbers in boxes represent PML and RARα exons.

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4.4 The reciprocal *RARα/PML* fusion

The reciprocal *RARα/PML* fusion generated in t(15;17) is present in 70–80% of APL cases (47). Two transcripts can be generated from the alternative RARα promoters, of which *RARα/PML* was the most common. Several different forms of *RARα/PML* were also found due to alternative breakpoints within the PML gene. There is no difference in ATRA sensitivity or clinical outcomes of patients who do or do not harbor the *RARα/PML* transcript. Patients with prolonged remissions of APL may express the *RARα/PML* transcript and not the *PML/RARα* transcript, suggesting that the small number of cells that harbor these genes may not have leukemogenic potential. Transgenic mice harboring the *RARα/PML* fusion did not develop leukemia, when crossed with *PML/RARα* mice, leukemia developed with greater frequency.

5. Other partner gene of RARα and reciprocal products of APL

In most of APL cases, a t(15;17) (q21;q22) translocation occurs fusing the RARα gene to the promyelocytic leukemia (PML) genes. More recently, variant chromosomal aberrations have been found involving chromosome 17q21, that fuse the same part of RARα to genes other than PML such as PLZF, NPM, NuMa and Stat5b (Table 6) (40).

5.1 The PLZF gene and its fusion with RARα

In particular, t(11;17) (q23;q21) is the most common, resulting from fusion between RARα and promyelocytic leukemia zinc finger (PLZF) genes (40); this extends the spectrum of morphological features associated with this leukemia. A previously reported from the European Working Party on APL found that 0.8% of APL patients harbored the t(11;17)(q23;q21) translocation. This translocation is associated with unfavorable prognosis and poor response to ATRA (52, 53) both in vivo and in vitro. Transgenic animals harboring *PLZF/RARα* developed a CML-like syndrome rather than APL.

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Table 6 APL and APL-like acute leukemia with a translocation involving RARα (17q21)(55)

Gene	Location	Name	Function	Sensitive to ATRA	Frequency
PML	15q22	Promyelocytic leukaemia	Signal transducer activ. transcript.	Yes	99%
PLZF	11q23	Promyelocytic leukaemia zinc finger	Morphogenesis (limbs, skeleton)	No	0.8%
NPM	5q35	Nucleophosmin	Ribosome biogenesis	Yes	<0.01%
NuMA	11q13	Nuclear mitotic apparatus	Chromatin compaction	Yes	<0.01%
Stat5b	17q11	Signal transducer activ. transcript.	Transcription factor for EPO, G-CSF, IL-2, IL-3, IL-7	No	<0.01%

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PLZF protein is localized to the nucleus in a pattern of 50 small nuclear speckles. PLZF is expressed in undifferentiated myeloid cell lines and at lower levels in more differentiated erythroleukemia, promyelocytic and monocytic cell lines as well as in peripheral blood mononuclear cells. PLZF expression may be important for the maintenance of hematopoietic stem cells and/or early progenitors, is down regulated with differentiation and may be re-expressed in monocytes. PLZF is a growth suppressor. The *PLZF/RARα* fusion did not colocalize with PML or de-localize PML from nuclear body structures, indicating that disruption of the PML NB is not required for the development of APL. In contrast, in t(15;17) and t(5;17) APL, PLZF is de-localized into a microspeckled pattern similar to that of *PML-RARα*, suggesting that de-localization of PLZF might be important in the pathogenesis of APL.

The reciprocal *RARα/PLZF* transcript links the ligand independent activation domain of RARα to the last seven PLZF zinc fingers. *PML/RARα* and *PLZF/RARα* have some similarities as aberrant receptors. Both fusion proteins can bind as homodimers to retinoic acid response elements (RARE). The reciprocal transcript encoding *RARα/PLZF* is consistently expressed in nearly all of the t(11;17)(q23;q21) patients. While PLZF represses gene transcription, *RARα/PLZF* activates it. *RARα/PLZF* activates expression of cyclin A2 and enhances cell growth. Hence t(11;17)(q23;q21) may be an ATRA and chemotherapy resistant disease due to the presence of two oncogenes. *PLZF/RARα* blocks the differentiation inducing action of ATRA, while *RARα/PLZF* may activate cell cycle regulators, blocking the anti-proliferative effects of ATRA. The *PLZF/RARα* mice did not achieve durable remission after ATRA treatment, although cells did show some evidence of differentiation, and leukemic cells from the mice treated with ATRA differentiated ex vivo. In mice harboring the *RARα/PLZF* protein develop a myeloproliferative syndrome and splenomegaly. When *PLZF/RARα* mice was crossed with *RARα/PLZF* mice, leukemic latency at 6 months and penetrance of 100% did not change. There was a phenotypic change in the leukemia that developed in the double-transgenic mice.

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5.2 NPM and NPM/RARα

In t(5;17)-associated APL, RARα is translocated to a region on chromosome 5q35 containing the ubiquitously expressed and evolutionarily conserved NPM gene. In hematological malignancies NPM is also fused to genes other than RARα.

The *NPM/RARα* fusion acts as a ligand dependent transcriptional activator when co-expressed with reporter genes containing RAREs, yet can act as a dominant-negative inhibitor of wild-type RARα through aberrant interaction with co-repressor molecules. Like other APL fusions, *NPM/RARα* could form homodimers, thus exposing the RARα moiety in such a way as to offer extra binding sites for co-repressors, necessitating pharmacological doses of ATRA to release them and recruit co-activators to the fusion proteins. *NPM/RARα* is expressed in a microspeckled pattern throughout the nucleus, and APL blasts from t(5;17) patients exhibited a normal PML NB configuration, supplying additional evidence that disruption of the PML NB is not required for the pathogenesis of APL.

A reciprocal *RARα/NPM* mRNA, leading to fusion between the A domain of RARα and the acidic domains, nuclear localization signal, and the rest of the C-terminus of NPM, the importance of this putative protein is unknown.

5.3 NuMA and its fusion with RARα

The t(11;17)(q13;q21) results in protein predicted to consist of the N-terminal globular and coiled-coil domains of NuMA, fused to the RARα domains B-F. *NuMA/RARα* localized to sheet-like nuclear aggregates in leukemic cells from the patient, but the PML-NB structures remained unperturbed. Introduction of C-terminal mutants of NuMA into cells completely disrupts mitosis; it is surprising that the presence of *NuMA/RARα* is even compatible with cell division.

The NuMA gene, located on chromosome 11q13, encodes a protein divided into two globular domains at either end of the protein, with a central coiled-coil region. The coiled-coil motifs probably mediate protein homo- and hetero-association. NuMA is a ubiquitously expressed protein involved in the completion of mitosis (54) and reformation of nuclei in the post-mitotic daughter cells. NuMA is regulated across the cell cycle by post-translational modifications. NuMA is a structural component of

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the cell that responds to cell cycle signals on cue rather than a controlling factor in cell proliferation. It seems unlikely that inhibition of NuMA function might contribute to oncogenesis.

The mechanism of the *NuMA/RARα* action is interference with nuclear receptor function. *NuMA/RARα* binds the RARE in vitro, and reporter gene experiments confirmed that the fusion protein inhibits transactivation by wild-type RARα in a similar fashion to other APL-associated fusion proteins. *NuMA/RARα* through its ability to form a complex with NuMA, may further inhibit RARα function by mis-localization of RARα cofactors in a nuclear compartment distinct from that occupied by the wild-type RARα. It is unknown whether a reciprocal *RARα/NuMA* transcript is expressed in this disease. *NuMA/RARα* forms a complex with the co-repressor SMRT which is released upon treatment with ATRA.

5.4 STAT5B/RARα fusion

Like RARα the STAT5b gene is localized on chromosome 17q21.1-21.2. The patient with the *STAT5b/RARα* fusion had an interstitial deletion of chromosome 17 leading to the creation of a fusion intron linking the STAT5b and RARα loci. As in the case of other forms of APL, the breakpoint in the RARα locus was within the same highly localized region 50 of the third coding exon. The *STAT5b/RARα* patient had ATRA-unresponsive disease (52) and leukemic blasts failed to respond to ATRA in vitro. No reciprocal RARα/STAT5b transcript was detected.

Signal transducer and activator of transcription (STAT) proteins are transcription factors that reside in the cytoplasm and translocate to the nucleus upon phosphorylation by JAK kinases, which are themselves activated by cytokine receptors. Seven different STAT proteins have been identified. STAT5b, like other STAT proteins, binds to the JAK kinase through an SH2 domain and then is itself tyrosinephosphorylated at a C-terminal residue. STAT5b can then homodimerize, migrate to the nucleus and bind to target DNA elements to activate transcription.

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6. Molecular-targeting therapy in APL

6.1 Treatment of APL with ATRA

All-*trans* retinoic acid (ATRA), the drug of first choice in the treatment of newly diagnosed APL (56, 57). ATRA was first introduced to clinical use for the treatment of APL in 1986. ATRA causes the reformation of PML bodies and promotes myeloid differentiation of APL blasts (44, 58). So, drug-induced differentiation is associated with restoration of normal nuclear structure in APL blasts and the mechanism for this involves degradation of the *PML/RARα* fusion protein (Figure 8). PML body restoration is also induced by As_2O_3 (arsenic trioxide), another drug routinely used for the treatment of APL (59).

The mechanisms of action of ATRA (60)

(1) The binding of ATRA to RAR receptors causes degradation of *PML/RARα* protein through the ubiquitin-protosome and caspase system, leading to restoration of terminal differentiation of promyelocytes.

(2) Exposure of APL cells to ATRA in vitro or in vivo induces relocalization of PML and restores the normal structure of PODs.

(3) Under the action of ATRA at pharmacological concentration (1 μM), CoR is dissociated from the repressive complex, whereas CoA (coactivator) is recruited to the complex. As a result, the repression of transcriptional activation of target genes is relieved and the differentiation of promyelocytes' process is restored (61).

ATRA induces differentiation in two major pathways.

(1) ATRA disassociates corepressor from the PML/RAR/RXR complex and recruits CoA(coactivator), leading to transcription of target genes.

(2) ATRA regulates the cAMP-PKA signaling pathway, which may also lead to the transcription activation and finally the differentiation of APL cells.

Besides, ATRA could modulate and degrade the *PML/RARα* oncoprotein.

6.2 Treatment of APL with arsenic compounds

Apart from the organic arsenicals (in which arsenic atoms are covalently bound to carbon-containing molecules), there are three main inorganic arsenic forms: red arsenic (As_4S_4, also known as 'realgar'), yellow arsenic (As_2S_3, also known as

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'orpiment'), and white arsenic (arsenic trioxide, As_2O_3), which is made by burning realgar or orpiment (62).

Treatment of APL by arsenic compounds represents a successful example of apoptosis induction therapy of acute leukemia. Arsenic was used for more than 500 years in traditional Chinese medicine (TCM). In the early 1970s, a group of investigators from Harbin Medical University in the northeastern region of China reported that a crude solution of arsenic trioxide (As_2O_3; ATO). Arsenic was viewed as both a therapeutic agent and a poison.

Mechanisms of As_2O_3 therapy

The kinetics and morphology of myeloid differentiation (57) are similar in patients with APL who have received therapy with RA or As_2O_3. The ability of ATO to induce high rates of CR in patients with relapsed APL provides an impetus for determining the specific mechanisms of action that mediate the partial differentiation and apoptosis of APL cells (44, 63-67) (Figure 8).

Ex vivo experiments revealed that lower doses of As_2O_3 induced partial myelocyte-like differentiation, which became terminal on the addition of cytokines or cyclic AMP. Morphologic changes indicating partial myeloid differentiation occur in vitro in the NB4 APL cell line following treatment with ATO at low concentrations (0.1-0.5 μM). After 10 days in the presence of ATO, the NB4 APL cell line demonstrates a decreased nuclear/cytoplasmic ratio, the appearance of cytoplasmic granules, the condensation of chromatin, and the partial disappearance of nucleoli. ATO also modulates the expression of cell surface differentiation antigens.

At high concentrations (>0.5 μM), ATO induces apoptosis, possibly by indirectly impairing H_2O_2 catabolism with induction of a decrease in mitochondrial membrane potential, release of cytocrome C, and activation and upregulation of caspases 1, 2, 3, and 8.

Toxic side effects

Arsenic, tasteless agent induces acute or chronic illness, mimicking common disorders such as haemorrhagic gastroenteritis, cardiac arrhythmia or psychiatric disease (68). But arsenic was never shown to be carcinogenic in animal models or

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cells in culture, so the mechanism by which it promotes tumor development is not understood. Surprisingly, arsenicals are still widely used to promote animal growth and vitality.

The patients can even develop retinoic acid syndrome (RAS) like symptoms such as fever, skin rash, edema, which were quickly relieved by steroid administration Other mild side effects in relapsed patients, such as fatigue, fever, edema, nausea, anorexia, diarrhea, emesis, headache, insomnia, cough, dyspnea, dermatitis, tachycardia, pain, hypokalemia, hypomagnesemia, and hyperglycemia. The most common events were abdominal pain, epistaxis, dyspnea, hypoxia, bone pain, thrombocytopenia, neutropenia, hypokalemia, and hyperglycemia. As_2O_3 treatment resulted in elevated plasma liver transaminase levels in seven cases and died of severe hepatic toxicity, in contrast to the mild liver dysfunction in about one third of the patients treated for relapsed APL. It was thus recommended that ATRA should be used as the first line drug for remission induction in newly diagnosed APL cases, while As_2O_3 can be either included into multidrug consolidation/ maintenance clinical trials after the first CR or used as a rescue for relapsed cases.

6.3 Treatment of APL by combining ATRA and As_2O_3

The combination of ATRA and arsenic in patients with newly diagnosed disease appears to achieve better remission induction and disease-free survival than with ATRA or ATO alone (69), and the combination of As_2O_3 and RA can induce remissions in relapse patients that are resistant to single therapies.

Three points may be worth mentioning with regard to the clinical effect of ATRA /As_2O_3 combination therapy. First, the beneficial effects were observed in a group of newly diagnosed APL, in contrast to a recent report on a trial of ATRA /As_2O_3 among relapsed APL patients where no obvious superiority was shown in the clinical outcome as compared with As_2O_3 monotherapy. Because most relapsed patients lost sensitivity to ATRA due to previous exposure, it would be hard to expect a full play of the synergism between ATRA and As_2O_3 in those patients. Second, ATRA /As_2O_3 induced earlier recovery of platelet counts, despite the fact that no obvious differences in the time course of recovery of coagulopathy were found among three groups. It is well known that the low platelet level in APL results from

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repression of the normal hematopoiesis and increased consumption destruction level because of the coagulopathy. The combination therapy relieves the repression of hematopoiesis more significantly than mono-therapy. Third, the side effects of combination therapy could be well controlled. Previous work showed that liver lesion was the most important toxic effect of As_2O_3 in newly diagnosed APL. The long term side effects of arsenic could be even decreased in the combination group.

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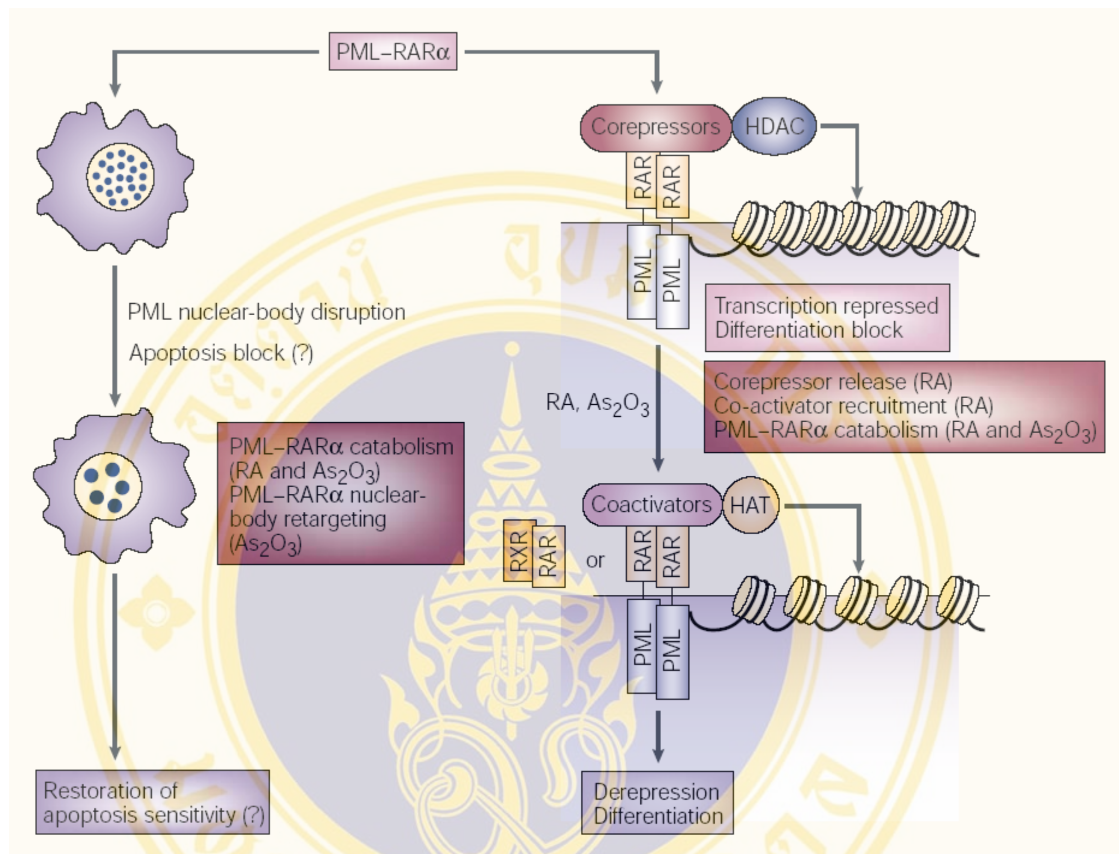

Figure 8 Two effects of retinoic acid and As_2O_3 on *PML/RARα* function. *PML/RARα* has dual effects. It not only disrupts PML nuclear bodies, which might result in apoptosis resistance (left), but it also blocks the transcription of RARα target genes (right). On the right, *PML/RARα* homodimers bind tightly to transcriptional corepressors, which attract histone deacetylases (HDACs). Treatment with retinoic acid (RA) dissociates corepressors from the *PML/RARα* fusion protein and turns it into an activator, allowing the recruitment of histone acetyltransferases (HATs) and gene transcription to occur. RA- or As_2O_3-induced *PML/RARα* catabolism also allows RAR-RXR to bind and activate transcription. On the left, RA or As_2O_3 induces the degradation of the fusion protein, allowing apoptosis to occur. (Zhu J., et al, 2002 (62))

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7. FMS-like tyrosine kinase 3 (*FLT3*) gene

7.1 Structure and expression of *FLT3*

The FMS-like tyrosine kinase 3 (FLT3), also called stem cell kinase 1 (STK1) or fetal liver kinase 2 (flk2), belongs to the group of class 3 receptor tyrosine kinases, together with other growth factor receptors such as c-Kit, PDGF-R, and *c-fms*, encodes a membrane-bound receptor tyrosine kinase (RTK) that has a crucial role in normal haematopoiesis (70, 71). The human *FLT3* gene is located on chromosome 13q12, contain 24 exons. Human FLT3 have two forms, a 158-160-kD a membrane-bound protein that is glycosylated at *N*-linked glycosylation sites in the extracellular domain and an unglycosylated 130–143- kD a protein that is not membrane bound. In normal human cells, expression of FLT3 occurs mainly in early myeloid and lymphoid progenitors, and not in erythroid cells, megakaryocytes or mast cells. *FLT3* messenger RNA has also been found in other lympho-hematopoietic organs, such as the liver, spleen, thymus and placenta. The FLT3 receptor tyrosine kinase is expressed on blast cells in most patients with acute myeloid leukemia (AML), and activating mutations of FLT3 have been detected in approximately 30% of these patients (72). Two distinct groups of *FLT3* mutations are most common (71): internal tandem duplications (ITDs) of the juxta membrane coding sequence in 20% to 27% of patients with AML and point mutations within the activation loop (A-loop) of the second tyrosine kinase domain (TKD) in about 7% of patients with AML (Figure 9).

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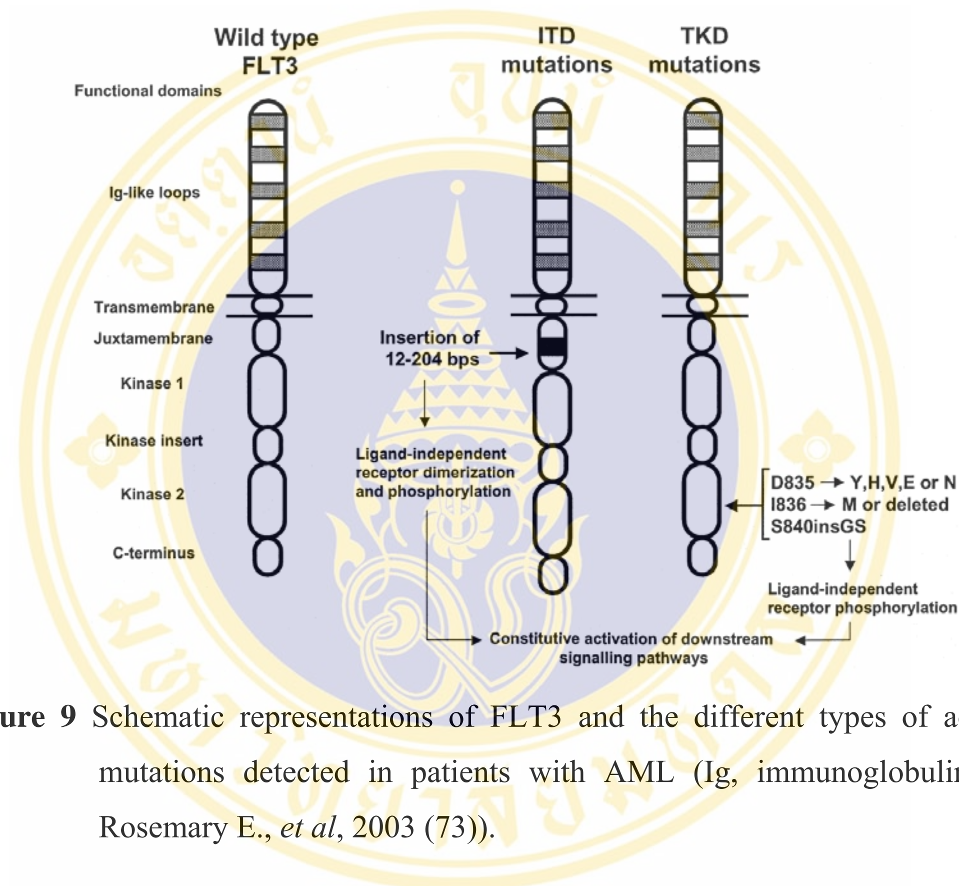

Figure 9 Schematic representations of FLT3 and the different types of activating mutations detected in patients with AML (Ig, immunoglobulin) (Gale Rosemary E., *et al*, 2003 (73)).

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7.2 *FLT3* gene mutation in hematopoietic malignancies

7.2.1 *FLT3* with internal tandem duplication (ITD)

The most common form of *FLT3* mutation is an internal tandem duplication (ITD) in exons 14 and 15 (previously known as exons 11 and 12), which occurs in 15-35% of patients with AML and 5-10% of patients with myelodysplasia (MDS). *FLT3*-ITD are formed when a fragment of the juxtamembrane domain coding sequence is duplicated and inserted in a direct head-to-tail orientation. The length of the ITD varies from 3 to ≥400 base pairs, and the reading frame of the transcript is always preserved, either by a faithful in-frame duplication or by the insertion of nucleotides at the ITD junction to maintain the original reading frame.

Most studies indicate that pediatric and adult AML patients with *FLT3* mutations have a poor prognosis. FLT3 has not been reported as a poor prognostic indicator in adults with AML over the age of 65, which may reflect the overall worse prognosis of this group compared to younger individuals.

7.2.2 Mutation in the tyrosine kinase domain (TKD)

The second most common type of *FLT3* mutation is a missense point mutation in exon 20 (previously known as exon 17) of the TKD. Mutations at aspartic acid 835 (D835) and isoleucine 836 (I836) in exon 20, in the second TK domain, were first independently identified in AML patients by Yamamoto et al (2001) (74) and Abu-Duhier et al (2001) (75) and have now been reported by many other groups. They include at least six different substitutions within the D835 codon leading to missense mutations, predominantly tyrosine and histidine, less frequently valine, glutamate and asparagine, and mutation of I836 to methionine (Figure 9). The complete deletions of I836, insertion of nucleotides and complex changes have also been detected, but the sequence always remains in frame.

TKD mutations occur in patients with AML (5-10%), MDS (2-5%) and ALL (1-3%), and the most common nucleotide substitution (GAT > TAT) changes an aspartic acid to tyrosine (D835Y) (71, 74). All TKD mutations maintain the same open reading frame. A single patient will occasionally have both an ITD and TKD point mutation of *FLT3*, but most patients have only one type of *FLT3* mutation. In total, ~25-45% of patients with AML will have some form of mutation in *FLT3*. The point

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mutations of codons 835/836 within the tyrosine kinase domain of FLT3 were also found in infant and childhood acute lymphoblastic leukemia (ALL).

8. *PML/RARα* and *FLT3* in APL

The association of FLT3 mutations with specific cytogenetic categories or other acquired mutations is of considerable biological interest as it may reveal clues to co-operating abnormalities leading to the transformed phenotype. A high frequency of *FLT3*-ITDs (30-39%) has been observed in patients with t(15;17) and, when combined with TKD mutations, nearly half the APL patients studied carry a *FLT3* mutation (76, 77). The ITD in particular appear to be associated with M3 variant morphology (65-80% are ITD) (78), together with hyperleucocytosis and the presence of the short breakpoint cluster region 3 (bcr3) *PML/RARα* isoform (76, 77, 79), indicating that these features may have a common causative pathology.

9. A model of APL with FLT3 mutation is responsive to retinoic acid and a receptor tyrosine kinase inhibitor

RTK-based, experimental anticancer strategies are now undergoing clinical evaluation for example, drugs that target FLT3 in AML (80).

The coexpression of an activated *FLT3* allele accelerates the appearance of leukemia in mice *MRP8 PML/RARα* mouse models that express a *PML/RARα* transgene and that inhibiting *FLT3* in combination with using ATRA to neutralize the effect of *PML/RARα* may have a future role in the treatment for APL patients with FLT3 mutations (81).

ATRA alone caused the differentiation of leukemic blasts into numerous mature neutrophils. ATRA caused some differentiation, but its major effect was to rapidly reduce leukemic cell mass.

A tyrosine kinase inhibitor able to block FLT3 activity had only a modest effect when used as a single agent but that it synergized with ATRA to rapidly cause disease regression. The tyrosine kinase inhibitor altered the effect of ATRA.

Some data indicate that APL patients with FLT3 mutations may benefit from combination therapy with all-*trans* retinoic acid plus an FLT3 inhibitor (82).

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CHAPTER IV
MATERIALS AND METHODS

1. Materials

1.1 Subjects and samples

The subjects included in the study were 609 Thai adult AML patients who attended Siriraj Hospital and affiliated hospitals. AML was diagnosed by blood smear and bone marrow examination. DNA and RNA were obtained from bone marrow or blood samples at time of diagnosis in these AML patients. The patients were aged between 13 and 86 years old. This study was a part of a large Leukemia Project previously approved by the Ethical Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University.

Of 609 AML patients, 80 APL cases were identified for subsequent molecular analysis. For immunophenotypic comparison, various types of AML samples with different karyotypes were also included in this studied.

For normal controls, peripheral blood mononuclear cells from 20 healthy volunteers working at the Faculty of Medicine Siriraj Hospital, Mahidol University were used.

1.2 Antibodies

The designation, cell type and antigens of the monoclonal antibodies used for immunophenotyping of acute leukemia are listed in Table 7.

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Table 7 Relevant antigens in immunophenotyping of acute leukemias (15).

Designation	Cell type	Antigen
CD2	T-cells, most NK cells	Erythrocyte-rosette receptor
CD3	Surface expression on mature T cells, cytoplasmic	Assciated with the T cell receptor, mediates signal expression in immature T cell transduction
CD5	Thymocytes, mature T cells, subpopulation of B cells	Linked to T cell proliferation
CD7	T cells, NK-cells, supopulation of immature myeloid cells	40 kDa protein
CD10	c-ALL, lymphatic precursor cells, neutrophils subset of 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 cell	Iron 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	Monocyte, myeloid precursor cells	67 kDa glycoprotein neutrophils
CD34	Myeloid and lymphoid precursor cells	105-120 kDa glycoprotein
CD41a	Platelets, megakaryocytes	Glycoproten 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 precursor 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 (Gly A)	Erythrocytes, erythroblasts and erythoid 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	Myeloperoxydase including immature myeloid cells

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1.3 Cell lines

NB4 cells

Human promyelocytic cell line with t(15;17) carrying L type of *PML/RAR*α fusion gene were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.5% penicillin and streptomycin in the plastic flask at 37ºC in humidified air plus 5% CO_2 (25).

1.4 Oligonucleotide primers

(1) *PML/RAR*α

The breakpoint regions of *PML* and *RAR*α were shown in Figure 10 (A). In this study, two pairs of primers were designed to detect *PML/RAR*α fusion gene. Figure 10, B illustrates the location of primers used to detected distinct types of *PML/RAR*α gene variants. The mRNA sequences of *PML* and *RAR*α gene were obtained from GenBank data base under the accession number NC_000015 and AH007261, respectively.

The primer pairs used to detect *PML/RAR*α fusion gene in the first round were M2, M4, R5 and R8 (49). The relative positions of these primers are shown in Figure 10 B. The locations of seven primer pairs designed for amplification of *PML/RAR*α fusion gene are shown in Figures 11, 12 and 13.

β-actin primers were use as internal control. The following primer sequences were used: Forward: 5'- CCTTCCTGGGCATGGAGTCCTG-3'

Reverse: 5' -GGAGCAATGATCITGATCTTC -3' (49).

(2) *FLT3*

Previously published primers used for PCR amplification and sequencing reaction were 11F, 12R, 17F and 17R.

The nucleotide sequences of the primer pairs used in this study are as follow:

11F 5’-GCAATTTAGGTATGAAAGCCAGC-3’

12R 5’-CTTTCAGCATTTTGACGGCAACC-3’

17F 5’-CCGCCAGGAACGTGCTTG-3’

17R 5’-GCAGCCTCACATTGCCCC-3’

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The primers were purchased through custom synthesis service from BioService Unit, National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

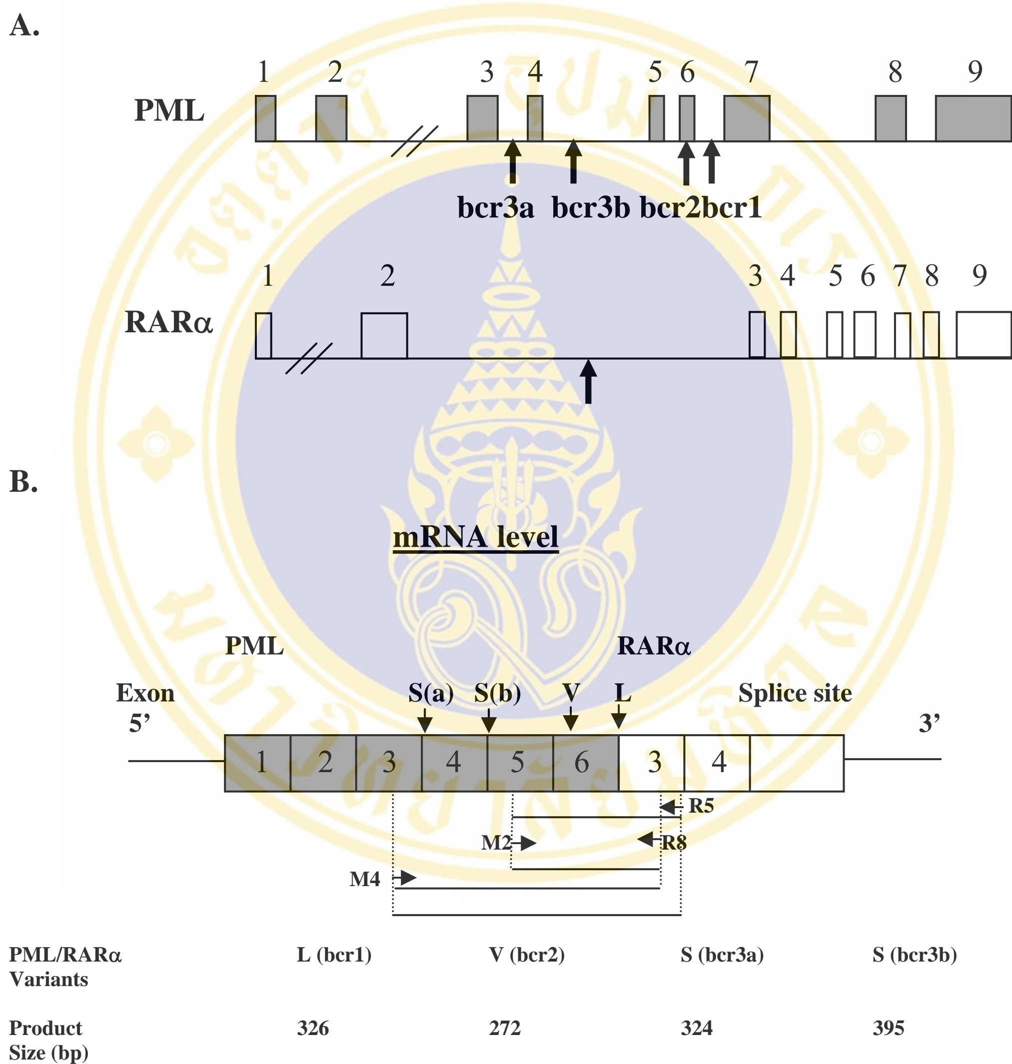

Figure 10 A. Diagram of *PML* and *RARα* loci. The breakpoint regions are indicated by arrows. B. Schematic representations of the *PML/RARα* mRNA transcripts showing the locations of primers used for amplifications of bcr1, bcr2 and bcr3 type and the product sizes. The vertical arrows indicate the three breakpoint cluster regions of the PML gene as follows: S-form (bcr3); V-form (bcr2); and L-form (bcr1).

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GGAGGACTTC TTGTCCCCAG TGAGCTCGGA GCTTTGAGTC AGGGAGCCTG GACCTGTGAC 37800
CCTCCTGAAG AACAGGGGTC ACTCGAGCCT CGAAACTCAG TCCCTCGGAC CTGGACACTG

TTGGTGAGGC CCCAGGGATT AGGCCAACCA CAGGTCTGGG GTACCTGGCC CTGGGGCTGC 37860
AACCACTCCG GGGTCCCTAA TCCGGTTGGT GTCCAGACCC CATGGACCGG GACCCCGACG

TGCCTAGTCA TTTCTGACTC AATTTTCCCA ACTTTGCAGC CCGAGGAGGC AGAGAGAGTG 37920
ACGGATCAGT AAAGACTGAG TTAAAAGGGT TGAAACGTCG GGCTCCTCCG TCTCTCTCAC
                                  Exon 5
AAGGCCCAGG TTCAGGCCCT GGGGCTGGCT GAAGCCCAGC CTATGGCTGT GGTACAGTCA 37980
TTCCGGGTCC AAGTCCGGGA CCCCGACCGA CTTCGGGTCG GATACCGACA CCATGTCAGT
                         M2 →
GTGCCCGGGG CACACCCCGT GCCAGTGTAC GCCTTCTCCA TCAAAGGCCC TTCCTATGGA 38040
CACGGGCCCC GTGTGGGGCA CGGTCACATG CGGAAGAGGT AGTTTCCGGG AAGGATACCT

GAGGTAAGGT TCTCCCCAGC CCCAGCCTTC CCTCCTGAAG GCCTGAGTTC CACCAGGAAA 38100
CTCCATTCCA AGAGGGGTCG GGGTCGGAAG GGAGGACTTC CGGACTCAAG GTGGTCCTTT

GTGAGCTGGC ACTGGGGTGG GAATCCAGGC CTTCATCAGA TCAGCTCTTT GTGGGAAAGT 38160
CACTCGACCG TGACCCCACC CTTAGGTCCG GAAGTAGTCT AGTCGAGAAA CACCCTTTCA
```

Figure 11 Nucleotide sequence of exon 5 of *PML* gene. Exons are indicated in blocks. Underlined are sequences used to design primers. Arrows indicate 5' to 3' direction of the primers (primers M2). Complete sequence of the *PML* gene (accession number NC_000015) was retrieved from GenBank database.

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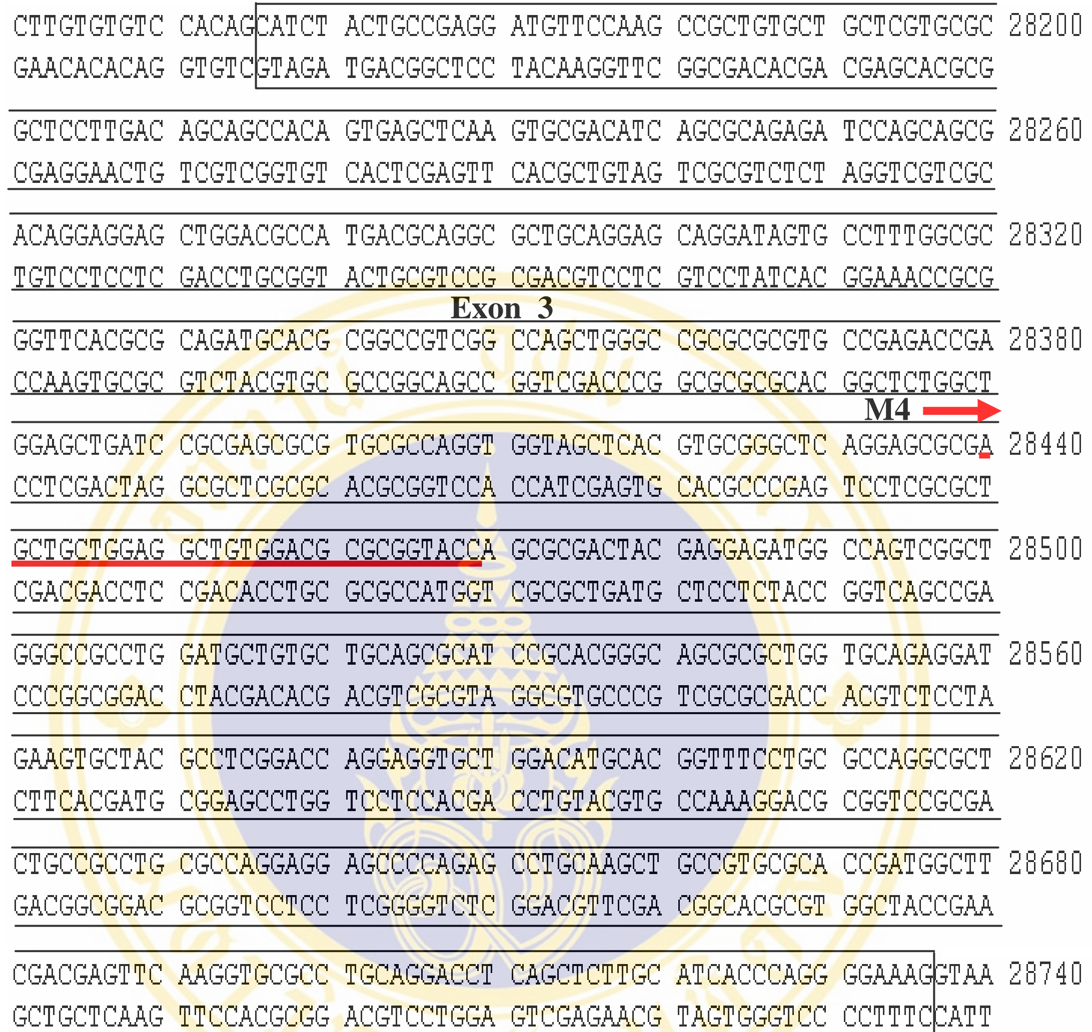

Figure 12 Nucleotide sequence of exon 3 of *PML* gene. Exons are indicated in blocks. Underlined are sequences used to design primers. Arrows indicate 5’ to 3’ direction of the primers (primers M4).

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GGAGCTTGGT ACTAAGGATG GCGACCTAGG TCTCTAACTG CCCCTCCCCT CTTCTCTCTC 2760
CCTCGAACCA TGATTCCTAC CGCTGGATCC AGAGATTGAC GGGGAGGGGA GAAGAGAGAG
                                 Exon 3
TAGCCATTGA GACCCAGAGC AGCAGTTCTG AAGAGATAGT GCCCAGCCCT CCCTCGCCAC 2820
ATCGGTAACT CTGGGTCTCG TCGTCAAGAC TTCTCTATCA CGGGTCGGGA GGGAGCGGTG
                                  R8
CCCCTCTACC CCGCATCTAC AAGCCTTGCT TTGTCTGTCA GGACAAGTCC TCAGGCTACC 2880
GGGGAGATGG GGCGTAGATG TTCGGAACGA AACAGACAGT CCTGTTCAGG AGTCCGATGG

ACTATGGGGT CAGCGCCTGT GAGGGCTGCA AGGTGAGTTG AAGGGGTCAT TGGGAAAGAC 2940
TGATACCCCA GTCGCGGACA CTCCCGACGT TCCACTCAAC TTCCCCAGTA ACCCTTTCTG
      R5
AGCTTGATGA GGTCAATGGG ATGTCCCCAC TTCTGTGTCC TGGGAGTGTG CAGTTGGGGG 3000
TCGAACTACT CCAGTTACCC TACAGGGGTG AAGACACAGG ACCCTCACAC GTCAACCCCC
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Figure 13 Nucleotide sequence of exon 3 of *RARα* gene. Exons are indicated in blocks. Underlined are sequences used to design primers. Arrows indicate 5' to 3' direction of the primers (primers R5 and R8). Complete sequence of the *RARα* gene (accession number AH007261) was retrieved from GenBank database.

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1.5 Other materials

Chemicals, instruments, enzymes and others are listsd in the Appendix.

2. Methods

2.1 Experimental strategy

The experimental steps began by identifying APL patients from a large AML patient cohort seen at Adult Hematology Clinic, Faculty of Medicine Siriraj Hospital. Mononuclear cells (MNC) were isolated from bone marrow (BM) or peripheral blood (PB) using Ficoll-hypaque density gradient centrifugation and collected for further nucleic acid isolation. cDNA was synthesized form RNA and used for nested RT-PCR reactions as described in section 2.5. Genomic DNA was used to study *FLT3* mutation as described in sections 2.6 and 2.7. Direct sequencing was performed to confirm *PML/RAR*α gene variants. The presence of *PML/RAR*α fusion gene was correlated with *FLT3* gene, immunophenotypes, and karyotypes. The experimental strategy is shown in Figure 14.

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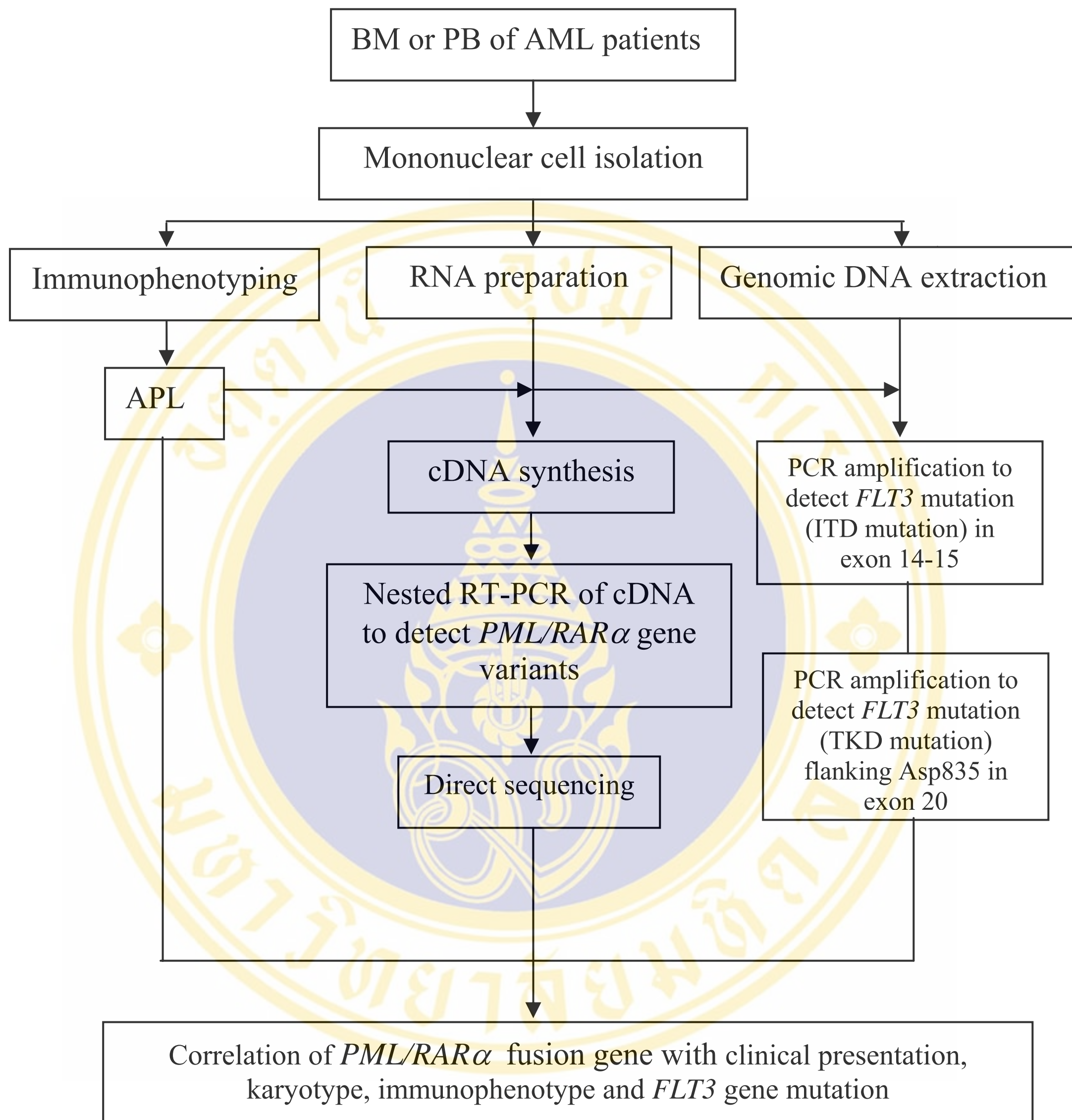

Figure 14 Experimental strategy used to study *PML/RARα* fusion gene and *FLT3* gene in this study.

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2.2 Separation of mononuclear cells

MNC were separated from heparinized bone marrow or blood samples for use in immunophenotyping and nucleic acid isolation**.** The samples were isolated by Ficoll-hypaque gradient centrifugation. The samples were diluted with three volumes of phosphate buffer saline (PBS) and layered on Lymphoprep™ (Fresenius Kabi Norge As, Oslo, Norway) and then centrifuged at 2,000 rpm for 20 minutes. The MNC layer was then collected; washed well twice with PBS, pelleted by centrifugation as well as subsequently used for immunophenotypic analysis and stored in a 1.5 ml microtube until use for nucleic acid preparation.

2.3 Immunophenotyping by Flow Cytometry

(1) BM aspirates or PB were immediately transported in sodium heparin tubes to the Flow Cytometry Laboratory, Department of Medicine, Siriraj Hospital.

(2) MNC were isolated using Ficoll-hypaque (15).

(3) For the detection of surface antigens, various combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP) labeled monoclonal antibodies against the following antigens (BD Biosciences, CA ,USA):CD2, CD3, CD5, CD7,CD10, CD11c, CD13, CD14, CD16, CD19, CD20, CD22 , CD33, CD34, CD41a, CD45, CD56, CD64, CD117, HLA-DR, glycophorin A (GlyA), nuclear terminal deoxynucleotidyl transferase (TdT), and myeloperoxidase (MPO) as listed in Table 7 were used. CD45 PerCP was added in all tubes.

(4) MNC were added to tubes that contained selected monoclonal antibodies and incubated for 15-30 minutes. The red blood cells were then lysed with FACS™ lysing solution (BD Biosciences, CA, USA). After 10 minutes of incubation, cells were washed with PBS, and fixed with 1% paraformaldehyde before flow cytometric analysis.

(5) For the detection of cytoplasmic MPO, and nuclear TdT antigens, the red blood cells were lysed with FACS™ lysing Solution and after 10 minutes of incubation, cells were washed and permeabilized for 10 minutes with FACS™ Permeabilizing Solution (BD Biosciences, CA, USA). Selected antibodies were added

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and incubated for 15 minutes. The cells were washed with PBS and fixed with 1% paraformaldehyde before flow cytometric analysis.

(6) Three-color flow cytometric immunophenotyping were performed on FACScalibur by collecting 10,000 ungated list mode events. An appropriate blast window was selected based on the combination of forward and side scatter characteristics as shown in Figure 15. 10,000 list mode events were collected for the blast window and cells were analysed within the selected window. An antigen is considered positive when at least 20% of the gated cells expressed that antigen.

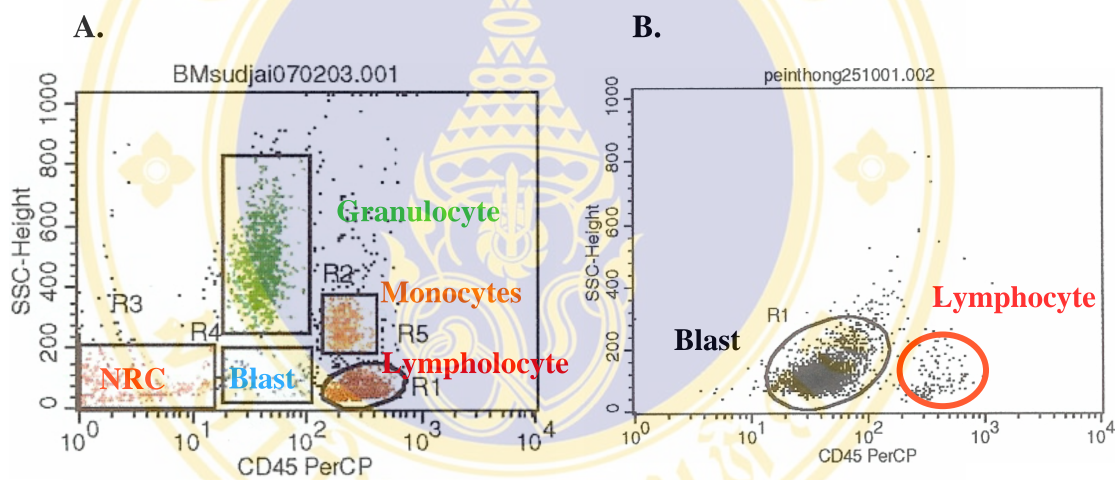

Figure 15 Immunophenotypic analysis of normal (A) and leukemic (B) bone marrow by CD45-side scatter analysis. When CD45 is combined with side scatter (SSC), which separates lineages based on cytoplasmic complexity, the bone marrow sample is readily separated into its cellular constituents. Infiltration of marrow by immature cells or blasts is more easily recognized on a CD45 versus side- scatter plot than on traditional forward and side-scatter gating.

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2.4 Nucleic acid isolation

Total RNA was isolated using a commercial modification of single-step guanidium thiocyanate-phenol chloroform, TRIzol® reagent (Invitrogen corporation, CA, USA), following the instructions of the manufacturer. Cells were mixed with 1 ml TRIzol® reagent and chloroform 200 µl, incubated at the room temperature for 2-3 minutes and then centrifuged at 12,000 rpm, for 15 minute, at 4˚C. Aqueous phase was transferred to another 1.5 ml microtube, and 0.5 ml of isopropyl alcohol was added and incubated at room temperature for 10 minutes, then centrifuge at 12,000 rpm for 10 minutes at 4˚C. RNA was washed by adding 0.5 ml of 75% ethanol in DEPC water, centrifuged 5 minutes at 7,500 rpm, 4˚C and let dried for 5-10 minutes. The dried RNA pellet was suspended in 10-50 µl of DEPC water depending on size of RNA pellet.

Genomic DNA was harvested from the remaining suspension by precipitation followed by a series of washes according to the supplier's recommendations.

cDNA was amplified from 1 µg of total RNA using AMV Reverse Transcriptase (Promega Corporation, USA) and random primers as the primers for first strand cDNA synthesis.

2.5 Analysis of *PML/RAR*α fusion gene by nested RT-PCR

PCR amplification by the nested primer technique was performed. For PCR amplification, a volume of 1 µL cDNA was diluted with 24 µL of PCR mixture containing 2.5 µl of 10x PCR buffer, 0.75 µl of 50 mM of $MgCl_2$, 0.5 µl of 10 mM dNTP, 0.125 of 1 U/µl of *Taq* DNA polymerase (Immolase, Bioline, Germany), 1 µl of 10 pmol of each M2 and R5 primers for L and V type and M4 and R5 for S type. β-actin was used as internal control.

After an initial denaturation at 95°C for 5 minutes, denaturation, annealing, and extension were performed on a Thermal Cycler (PCR Sprint Thermal Cycler, CA, USA) at 95°C for 1 minute, 55ºC for 1 minute, and 72°C for 1 minute respectively, for a total of 30 cycles. At the end, one microliter of the first PCR product was used for a second round of amplification for further 30 cycles using a nested primer (R8) as shown in Figure 13. PCR mixture contained 2.5 µl of 10x PCR buffer, 0.75 µl of 50

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mM of $MgCl_2$, 0.5 µl of 10 mM dNTP, 0.125 of 1 U/µl of *Taq* DNA polymerase (Immolase, Bioline, Germany), 1 µl of 10 pmol of each M2 and R8 primers for L and V type, M4 and R8 for S type, and 18.125 µl of sterile distilled water.

Finally, 5 µL of PCR mixture was run on a 2% SeaKem® LE agarose gel (BioWhitaker Molecular Applications, Rockland, USA) stained with ethidium bromide and the products were viewed on gel documentation machine.

2.6 Analysis of *FLT3* internal tandem duplication (ITD) mutation by PCR

For amplification of exon 14 to exon 15 of *FLT3* gene, PCR reaction was performed using a 25-µl PCR mixture which contained 2.5 µl of 10x PCR buffer, 0.5 µl of 50 mM of $MgCl_2$, 0.5 µl of 10 mM dNTP, 1 µl of 10 pmol of each 11F and 12R primers, 0.125 of 1 U/µl of *Taq* DNA polymerase (Immolase, Bioline, Germany), 17.375 µl of sterile distilled water, and 2 µl of 25 ng/µl of DNA template.

PCR was performed for 30 cycles in a Thermal Cycler (PCR Sprint Thermal Cycler, CA, USA). The detail of PCR cycles were as followed (i) denaturation at 94°C for 1 minute (5 minutes for the first cycle), (ii) annealing at 59°C for 1 minute, (iii) extension at 72°C for 1 minute (7 minutes for the last cycle).

The PCR products were detected by agarose gel electrophoresis. Five microliters of PCR products were mixed with 6x gel-loading buffer and the mixture was then applied into 2% agarose mini-gel slab, and electrophoresed at 100 volts for approximately 45 minutes. Fifty nanograms of 100 bp DNA ladder was also loaded in one well as a standard-size marker. The gel was stained in 2 µl/ml ethidium bromide solution and the products were viewed on gel documentation machine.

2.7 Analysis of *FLT3* tyrosine kinases domain (TKD) mutation by PCR-RFLP

Two primers (17F and 12R) were used for the amplification of exon 20 of *FLT3* gene which is a part of tyrosine kinase domain. PCR reaction contained 2.5 µl of 10x buffer, 0.5 µl of 50 mM of $MgCl_2$, 0.5 µl of 10 mM dNTP, 1 µl of 10 pmol of each 17F and 17R primers, 0.125 of 1 U/µl of *Taq* DNA polymerase (Immolase, Bioline, Germany), 17.375 µl of sterile distilled water, and 100 ng/µl of DNA template.

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PCR was performed for 30 cycles in Thermal Cycle (PCR Sprint Thermal Cycler, CA, USA). The detail 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).

The PCR products were digested with restriction enzyme *Eco*RV as previously described (74). The digestion reaction reaction was carried out in 20 µl which contained 1 µl of 5x *Eco*RV buffer, 0.25 µl of 1 U/µl *Eco*RV enzyme, 12.25 µl of sterile distilled water, and 10 µl of PCR products. The mixture was incubated at 37°C overnight. After complete digestion, the digested products were fractionated through 2.5% agarose gel electrophoresis, 100 bp DNA ladder was also used as a standard-size marker.

2.8 Sequencing of PCR products

2.8.1 Purification of PCR products

The PCR products that showed specific aberrant pattern in agarose gel electrophoresis were purified for DNA sequence analysis by using QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA, USA) following the manufacturer's instructions. Briefly, the gel containing DNA fragment were excised with a sharp scalpel and placed into microtube. The gel was weighted and its volume was estimated. Then, 3 volumes of QG buffer were added to the gel. The incubation was made at 50°C for 10 minutes until the gel splice was completely dissolved. After the complete dissolution of the gel, one gel volume of isopropanol was added. The solution containing DNA was applied to the QIAquick column and centrifuge at 10,000 g for 1 minute. After that, residual agarose was removed from the column by adding 0.5 ml QG buffer and centrifuged for 1 minute. DNA was washed by adding 0.75 ml of PE buffer and centrifuged again. After discarding the flow through, centrifugation was repeated once. The DNA was then eluted from the column by adding 30 µl of EB buffer and collected in to 1.5 ml microtube by centrifugation at 10,000 g for 1 minute. The concentration of the purified DNA was estimated from the band intensity by comparing to standard DNA markers, *Hae*III digested Øx174 DNA.

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2.8.2 Cycle sequencing

The purified PCR products were sequenced by using DYEnamic™ ET Terminator Cycle Sequening kit (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, England) following the manufacturer's instruction. The rection was made by mixing 4 µl terminater ready reaction mix, 5 pmol primer, 30 ng of purified PCR product (as DNA template), and distilled water to make up the volume to 10 µl. Using Thermal Cycler (PCR Sprint Thermal Cycler, CA, USA), the DNA template in the mixture was amplified for 30 cycles, each of which consisted of 95°C for 20 second, 50°C for 15 second, and 60°C for 1 minute.

2.8.3 Preparation of cycle sequencing product for loading

The cycle sequencing product was purified from the excessive dye terminators by precipitation with 10 µl of ammonium acetate and 60 µl of 95% ethanol, then centrifuged for 15 minutes at 12,000 g, the pellet was rinsed with 200 µl of 70% ethanol, centrifuged 12,000 g for 5 minute and dried in dark place for 10-15 minutes. The dried pellet was suspended in 10 µl of MegaBACE loading solution (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, England).

2.8.4 Analysis of DNA sequence

The sequencing product suspension was automatically injection for 75 seconds at 2kV and run 9 kV for 100 minutes. The sequence data was examined by MegaBACE™ Sequencing System 1000 (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, England) and analyzed by using OMIGA program version 2.0.

2.9 Cytogenetic analysis

Conventional chromosome analysis was done by banding methods of all AML samples at DNA center, Bangkok. The nomenclature of chromosome abnormalities was according to the International System for Human Cytogenetic Nomenclature (ISCN) 1995 (83).

2.10 Statistical analysis

The chi-square test was the statistical tools used to analyze differences in the distribution of variables among subgroups of patients.

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Value of a test result

Test result are not perfect. They must be properly interpreted in the light of analytical performance, which includes accuracy and precision, and clinical performance, which varies with sensitivity, specificity and predictive value.

1. Sensitivity

The sensitivity of test refers to its ability to show positive results in patients.

$$\text{Sensitivity (\%)} = \frac{TP}{TP + FN} \times 100$$

Where TP = Number of true positives (diseased persons detected by the test)
and FN = Number of false negatives (diseased persons not detected by the test)

2. Specificity

The specificity refers to the percentage of negative results among people who do not have the disease.

$$\text{Specificity (\%)} = \frac{TN}{FP + TN} \times 100$$

Where TN = Number of true negatives (non-diseased persons negative to test)
and FP = Number of false positives (non-diseased persons positive to test)

3. Positive Predictive Value (PPV)

PPV is the probability that a person has the disease given a positive test result.

$$\text{PPV (\%)} = \frac{TP}{TP + FP} \times 100$$

4. Negative Predictive Value (NPV)

NPV is the probability of no disease among patients with a negative test.

$$\text{NPV (\%)} = \frac{TN}{FN + TN} \times 100$$

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CHAPTER V
RESULTS

1. Classification of AML according to the FAB and WHO Classification

A total of 609 samples of AML patients were classified by FAB classification as shown in Table 8. 13.1% of AML were categorized as M3 subtype. AML-M0, M1, M2, M4, M5, M6 and M7 were found in 2.0, 24.6, 31.0, 17.7, 8.2, 3.1 and 0.2% of all AML cases, respectively.

Table 8 Classification of 609 Thai AML patients according to the FAB classification

FAB type	N*	% of Total
M0	12	2.0
M1	150	24.6
M2	189	31.0
M3	80	13.1
M4	108	17.7
M5	50	8.2
M6	19	3.1
M7	1	0.2

* N; Number of cases

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609 AML were also classified by the recent WHO classification into four types with recurrent genetic abnormalities; AML with t(8;21)(q22;q22) (8.2%), AML with inv(16)(p13q22) (1.8%), AML with t(15;17)(q22;q21) (2.6%) and AML with 11q23 abnormalities (1.3%) as shown in Table 9.

Table 9 WHO classification of 609 Thai AML patients in this study.

WHO classification	**Number of cases (% of total)**
AML with t(8;21)(q22;q22)	50 (8.2)
AML with t(15;17)(q22;q21)	16 (2.6)
AML with inv(16)(p13q22)	11 (1.8)
AML with 11q23 abnormalities	8 (1.3)

Remarks 524 (86%) of 609 AML had karyotypes as follow:

Normal karyotype	302 (49.6%) cases
No metaphase	53 (8.7%) cases
trisomy 8	23 (3.8%) cases
monosomy 7	17 (2.8%) cases
t(9;22)	12 (1.95%) cases
trisomy 21	8 (1.3%) cases
trisomy 11	5 (0.8%) cases
other	104 (17.1%) cases

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The demographic data of Thai APL were shown in Table 10. More female were found in APL cases than male and the median age was 41.5 years. The median WBC was 26.4 x10^9/L with the median Hb of 8.1 g/dL and the median platelets of 34 x10^9/L, respectively.

Table 10 Demographic data of 80 Thai APL patients.

Parameter	APL	non-APL
Number of cases (%)	80 (13.1)	529 (86.9)
Female: Male	52:28	263:266
Median age (year)	41.5	42.0
Age range (year)	15-84	13-83
Age > 60 (%)	33.3	23.1
Median WBC (x10^9/L) (Range)	26.4 (0.6-306.1)	34.7 (0.3-569.9)
Median Hb (g/dL) (Range)	8.1 (1.3-14.5)	7.8 (1.4-14.0)
Median Plt (x10^9/L) (Range)	34.0 (6.0-663.0)	45.0 (4.0-1149.0)

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2. Incidence of *PML/RARα* fusion gene

A total of 80 samples were screened for the *PML/RARα* fusion gene (Table 11). The *PML/RARα* fusion gene was found in 62 of total 80 APL cases (77.50%). *PML/RARα* was detected in all cases with t(15;17) identified by karyotypic analysis. Moreover, 46 out of 64 cases without t(15;17) were also identified to have *PML/RARα* by molecular analysis.

Table 11 Incidence of *PML/RARα* in APL patients

***PML/RARα* analysis**	**APL**	
	Presence of t(15;17) (n=16)	**Absence of t(15;17) (n=64)**
***PML/RARα* positive (%)**	16 (100)	46 (71.9)
***PML/RARα* negative (%)**	0 (0)	18 (28.1)

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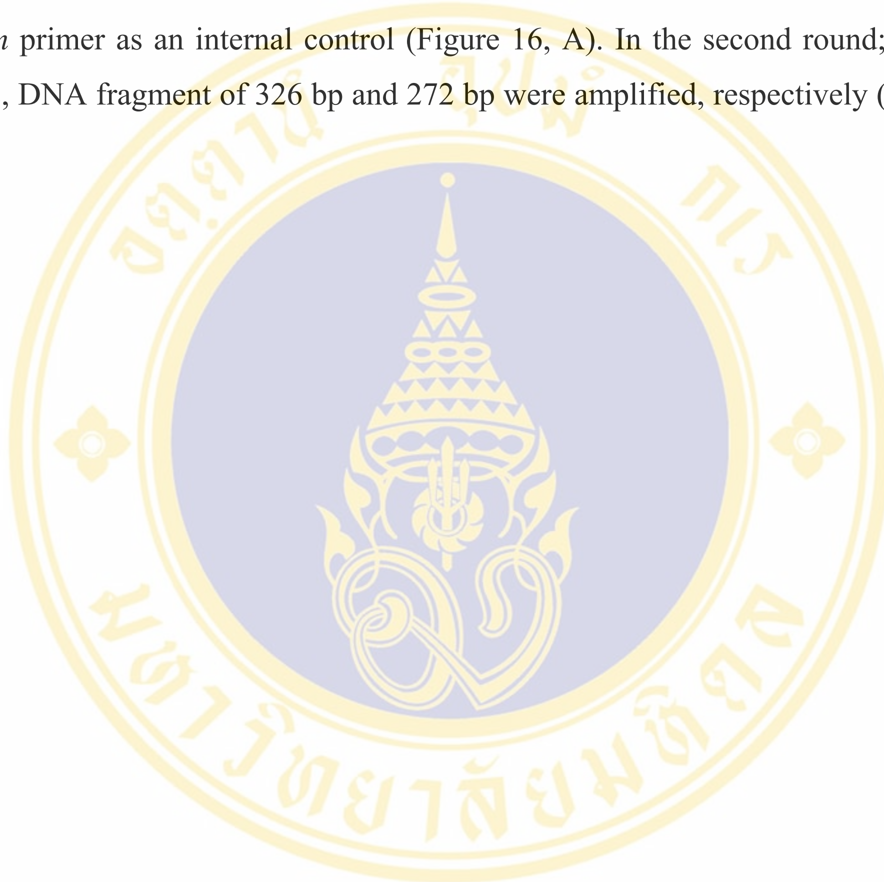
3. Characterization of *PML/RARα* fusion gene variants

The *PML/RARα* fusion gene of three different bcrs were PCR amplified and the nucleotide sequences were determined. Primer M2 derived from the *PML* exon 5 and primer R8 from *RARα* exon 3 were used for the amplification of bcrl and bcr2 *PML/RARα* mRNA junctions as shown in Figures 17. A first cycle of PCR used the *β-actin* primer as an internal control (Figure 16, A). In the second round; for bcr1 and bcr2, DNA fragment of 326 bp and 272 bp were amplified, respectively (Figure 16, B).



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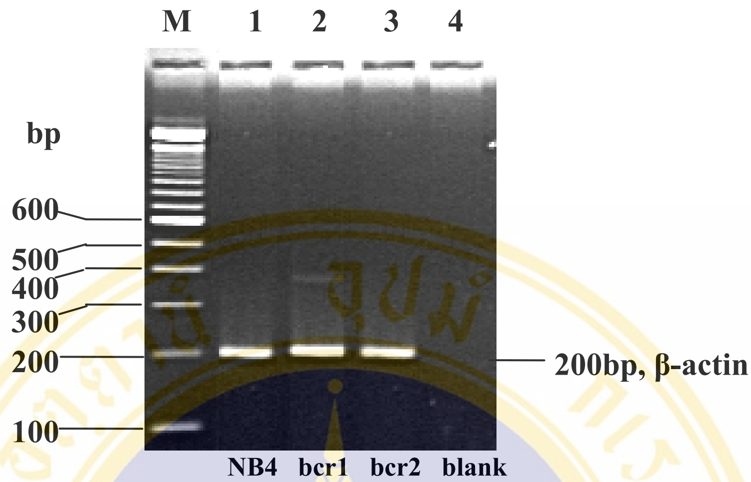
A.



B.

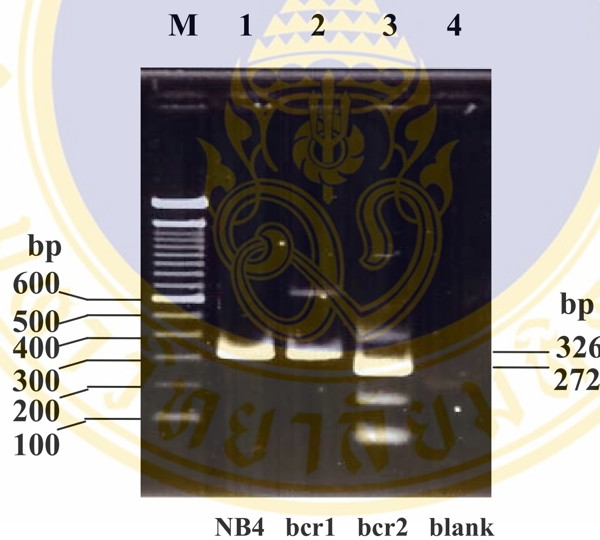


Figure 16 Representative results of the nested RT-PCR analysis of *PML/RAR*α isoforms. (A) RNA from NB4 cell line (lane 1); positive control for bcr1, two APL patients (lane 2 and 3), and H_2O (lane 4) for negative control were amplified. Primers M2, R5 and β-actin were used as a first cycle of PCR; then a second round of amplification was performed by using R8 as nested primer. Amplification of 200 bp of the β-actin gene as internal control was shown after the first round of nested RT-PCR. (B.) In the second round; for bcr1, DNA fragment of 326 bp were amplified, whereas band of 272 bp was observed for bcr2. Lane M represents 100 bp DNA ladder used as a standard-size marker.

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The *PML/RARα* bcr1 sequence in Figure 17 shows the bcrl junction (breakpoint of bcr1) at PML intron 6 and RARα exon 3 internal splicing. The nucleotide sequence came from the PCR amplification of the 326 bp *PML/RAR α* fragment.

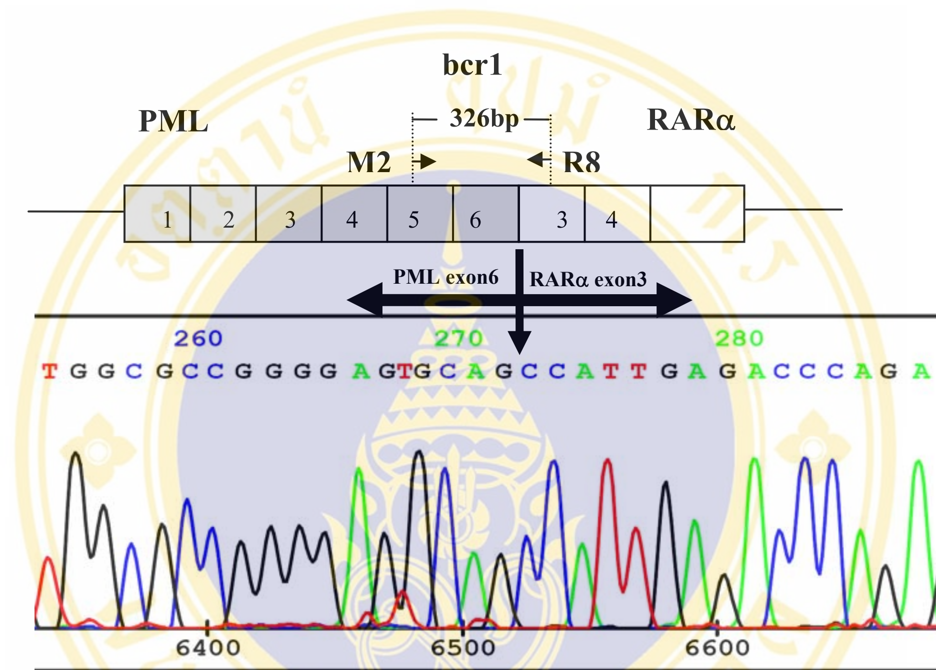

Figure 17 *PML/RARα* mRNA junctions. Upper diagram: schematic representation of *PML/RARα* bcr1 mRNA junctions. Gray boxes indicate PML exons and white boxes indicate RARα exons, (exon numbers are in the boxs). Lower diagram: schematic representation of the exon assembly predicted from the nucleotide sequence of the 326 bp bcrl *PML/RARα* mRNA PCR amplification product from the indicated APL case. The sequence of the *PML/RARα* mRNA junctions is shown below the arrow. The arrows indicate the bcrl junction and RARα exon 3 internal splicings.

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The *PML/RARα* bcr2 sequence in Figure 18 shows the bcr2 junction (PML exon 6) and RARα exon 3 internal splicing. The nucleotide sequence came for the PCR amplification of the 272 bp *PML/RARα* fragment.

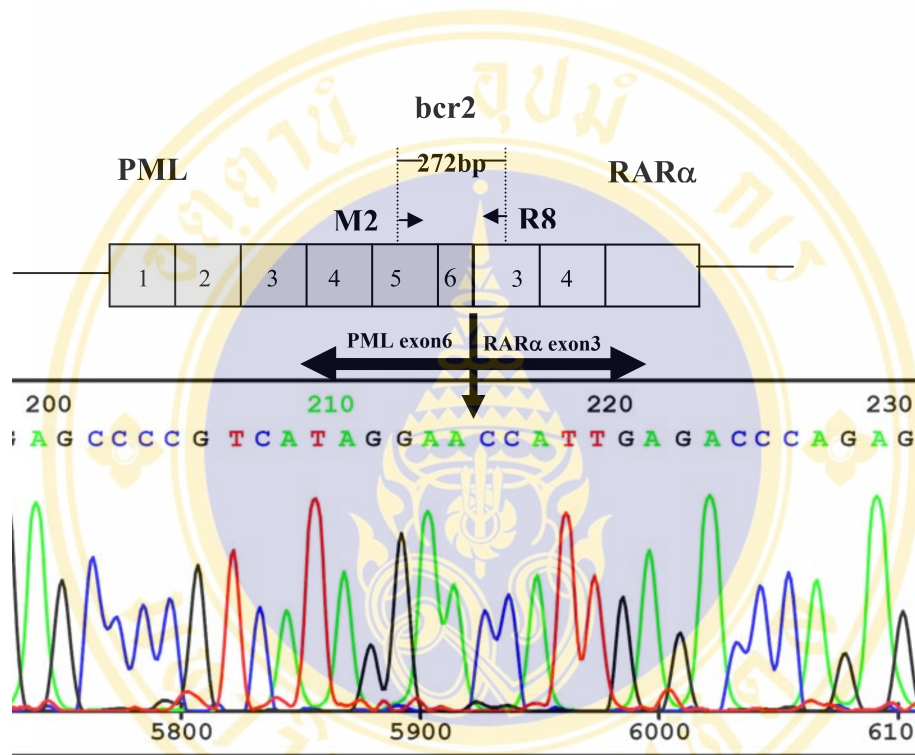

Figure 18 *PML/RARα* mRNA junctions. Upper diagram: schematic representation of *PML/RARα* bcr2 mRNA junctions. Exon numbers and symbols are the same as in Figures 17. Lower diagram: schematic representation of the exon assembly predicted from the nucleotide sequence of the 272 bp bcr2 *PML/RARα* mRNA PCR amplification product from the indicated APL case. The sequence of the *PML/RARα* mRNA junctions is shown below the arrow. The arrows indicate the bcr2 junction and RARα exon 3 internal splicings.

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The bcr3 variant of *PML/RARα* gene has two isoform, bcr3a and bcr3b. Primer M4 derived from PML exon 3 and primer R8 were used for amplification of bcr3 *PML/RARα* mRNA junctions. Agarose gel electrophoresis of bcr3 was shown in Figures 19.

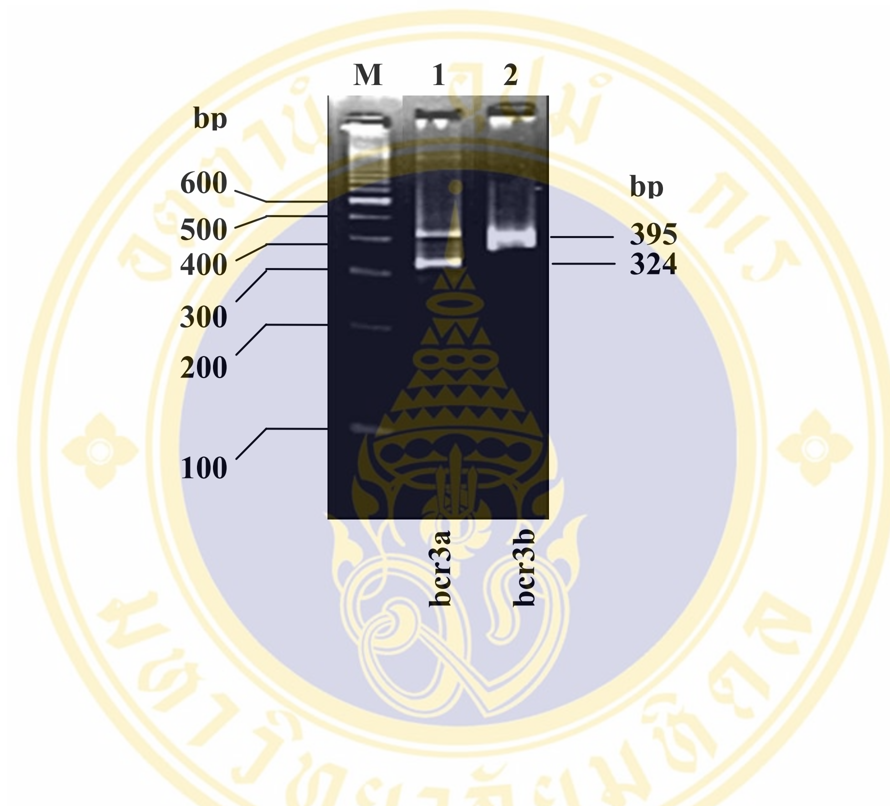

Figure 19 Representative results of nested RT-PCR analysis of *PML/RARα* isoforms. RNA from two APL patients (lanes 1 and 2) were amplified. Primers M4, R5 and β-actin were used as a first cycle of PCR; then a second round of amplification was performed by using R8 as nested primer. In the second round; for bcr3a, DNA fragment of 324 bp was amplified, whereas band of 395 bp was observed for bcr3b. Lane M represents molecular markers.

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The sequence of bcr3a was shown in Figure 20. The *PML/RARα* bcr3a mRNA involves breakpoint within PML intron 3 and RARα exon 3 internal splicing.

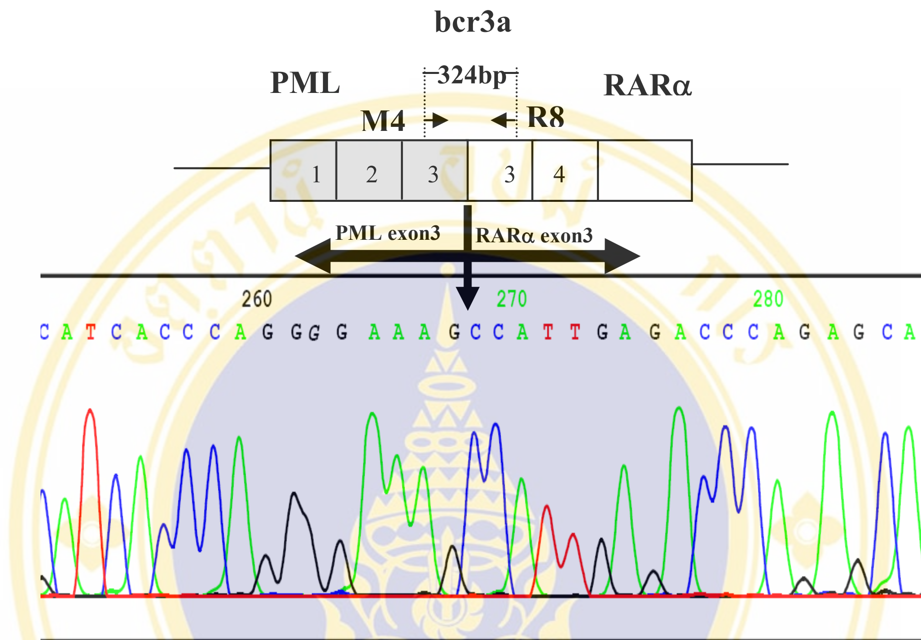

Figure 20 *PML/RARα* mRNA junctions. Upper diagram: schematic representation of *PML/RARα* bcr3a mRNA junctions. Exon numbers and symbols are the same as in Figures 17. Lower diagram: schematic representation of the exon assembly predicted from the nucleotide sequence of the 324 bp bcr3a *PML-RARα* mRNA PCR amplification product from the indicated APL case. The sequence of the *PML/RARα* mRNA junctions is shown below the arrow. The arrows indicate the bcr3a junction and RARα exon 3 internal splicings.

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The *PML/RARα* mRNA junctions and the sequence of bcr3b were shown in Figure 21. The bcr3b involves the intron 4 breakpoints. The *PML/RARα* bcr3b nucleotide sequence came from PCR amplification of the 395 bp *PML/RARα* fragment.

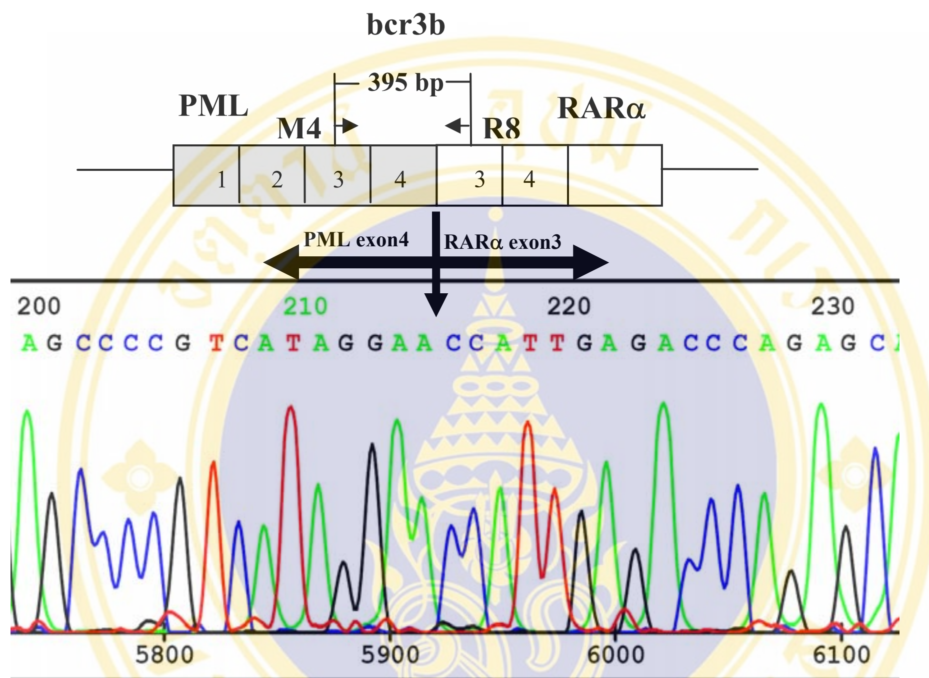

Figure 21 *PML/RARα* mRNA junctions. Upper diagram: schematic representation of *PML/RARα* bcr3b mRNA junctions. Exon numbers and symbols are the same as in Figures 17. Lower diagram: schematic representation of the exon assembly predicted from the nucleotide sequence of the 395 bp bcr3b *PML/RARα* mRNA PCR amplification product from the indicated APL case. The sequence of the *PML/RARα* mRNA junctions is shown below the arrow. The arrows indicate the bcr3b junction and RARα exon 3 internal splicings.

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Agarose gel electrophoresis of all isoforms were shown in Figures 22. PCR products and markers were visualized by ethidium bromide staining.

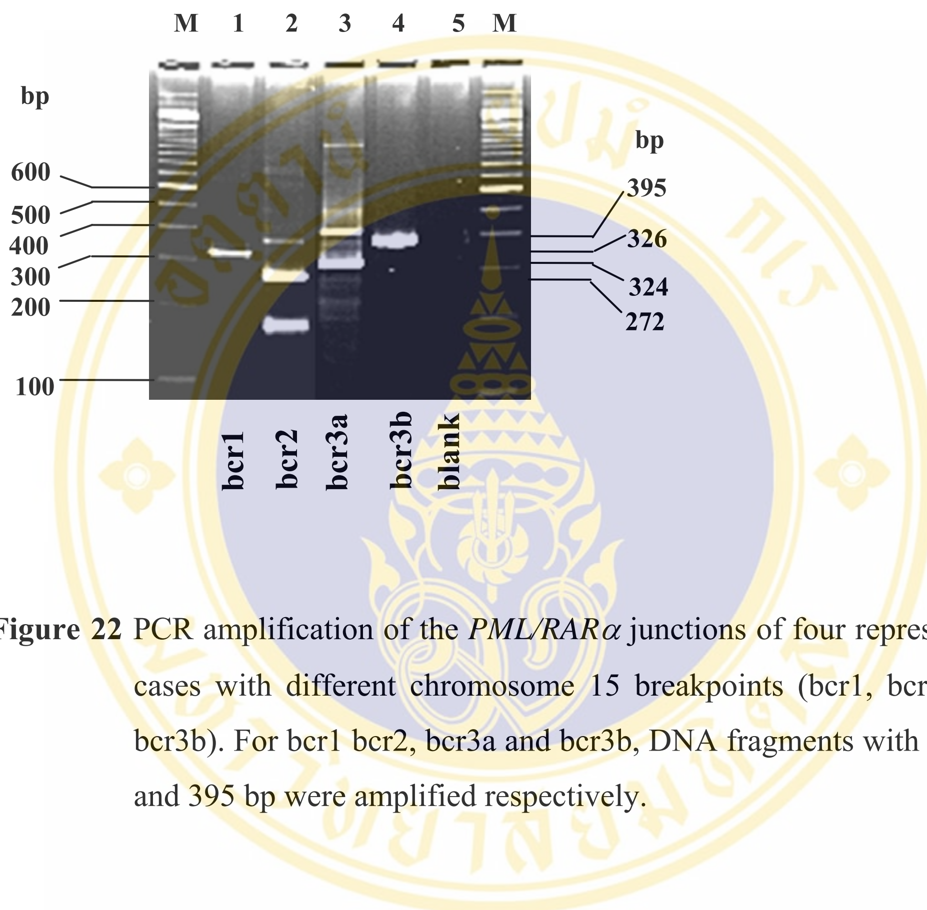

Figure 22 PCR amplification of the *PML/RARα* junctions of four representative APL cases with different chromosome 15 breakpoints (bcr1, bcr2, bcr3a, and bcr3b). For bcr1 bcr2, bcr3a and bcr3b, DNA fragments with 326, 272, 324 and 395 bp were amplified respectively.

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4. Frequency of different types of *PML/RARα* variants

L type (bcr1), V type (bcr2) and S type (bcr3) were present in 57 cases (71.3%), 3 cases (3.7%) and 2 cases (2.5%) of our APL cases, respectively (table 12). The bcr1 was the most commonly found in APL patients (71.3% of APL cases) as shown in Figure 23.

Table 12 Frequency of *PML/RARα* fusion gene in Thai adult APL patients

Type of *PML/RARα* variants	APL diagnosed by FAB					
	Presence of t(15;17) (n=16)		Absence of t(15;17) (n=64)		Total cases (n=80)	
	N	%	N	%	N	%
bcr1	13	81.3	44	68.8	57	71.3
bcr2	2	12.5	1	1.5	3	3.7
bcr3	1	6.2	1	1.5	2	2.5
Negative	0	0	18	28.2	18	22.5

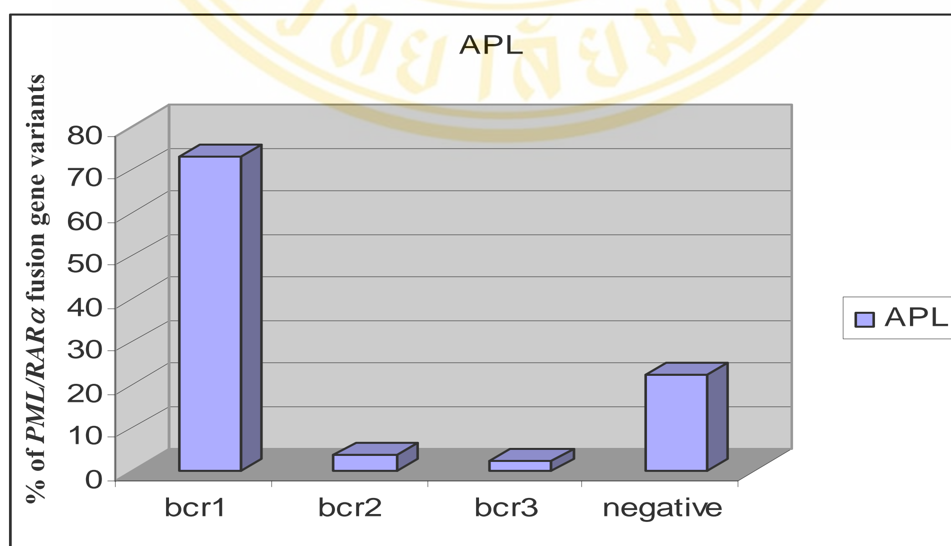

Figure 23 Type of *PML/RARα* fusion gene in Thai adult APL patients

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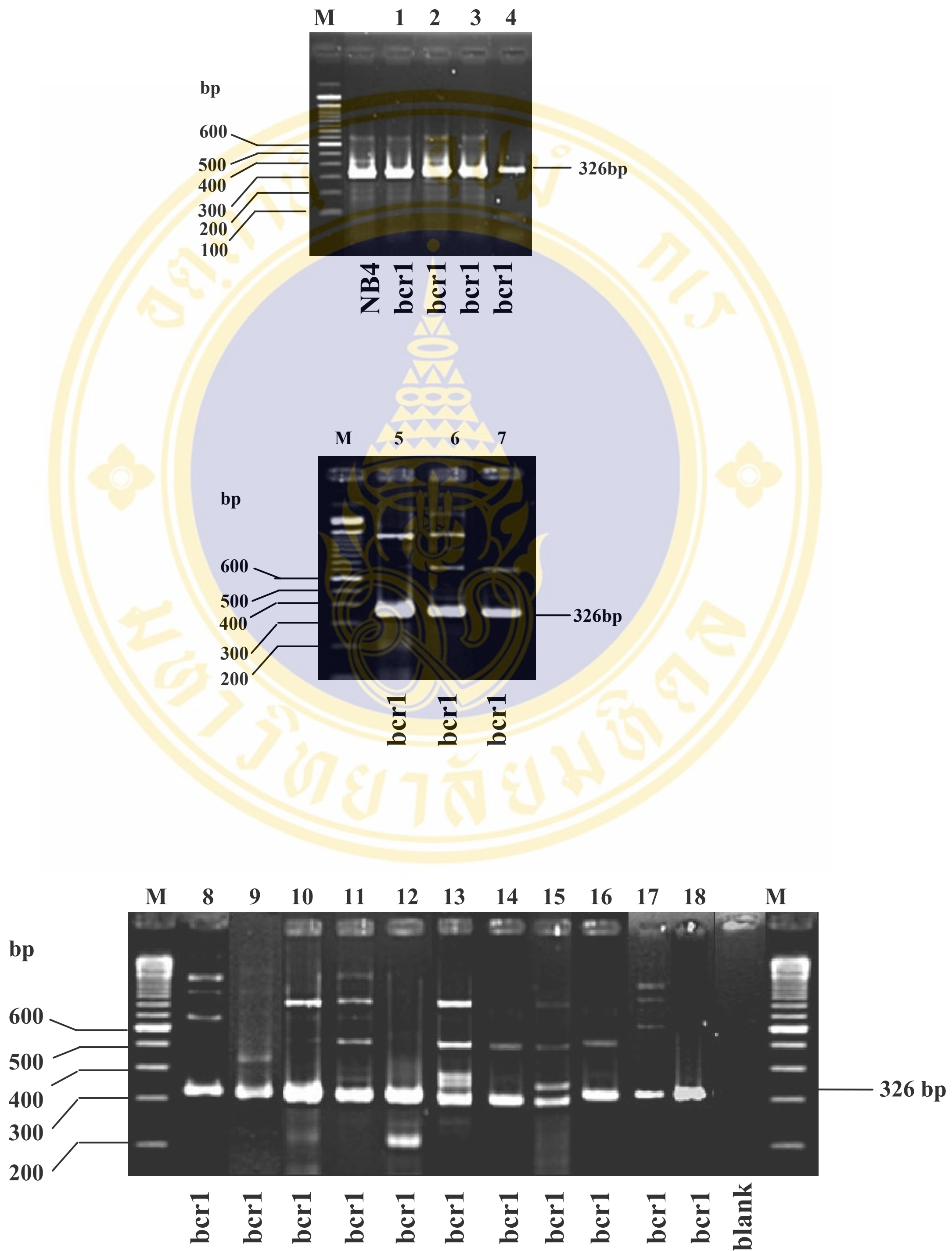
Agarose gel electrophoresis of PCR products of 57 cases with bcr1 and 3 cases with bcr2 are shown in Figure 24.



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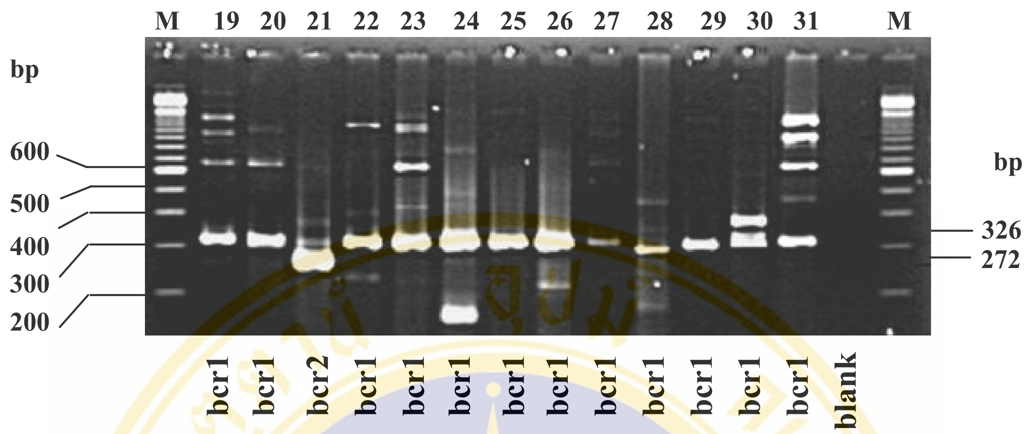

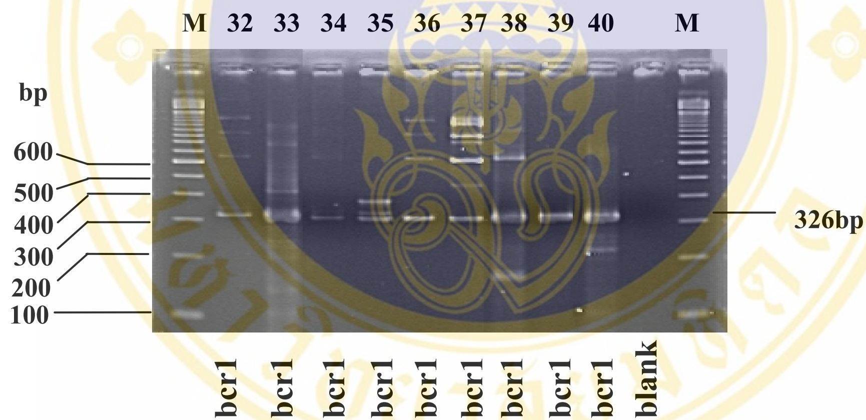

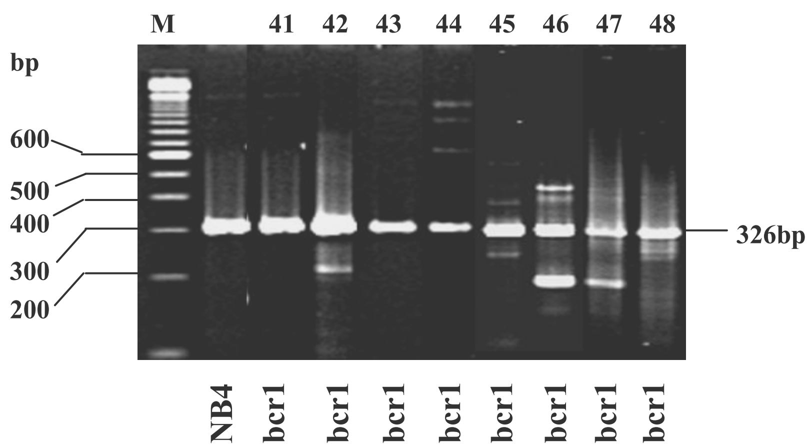

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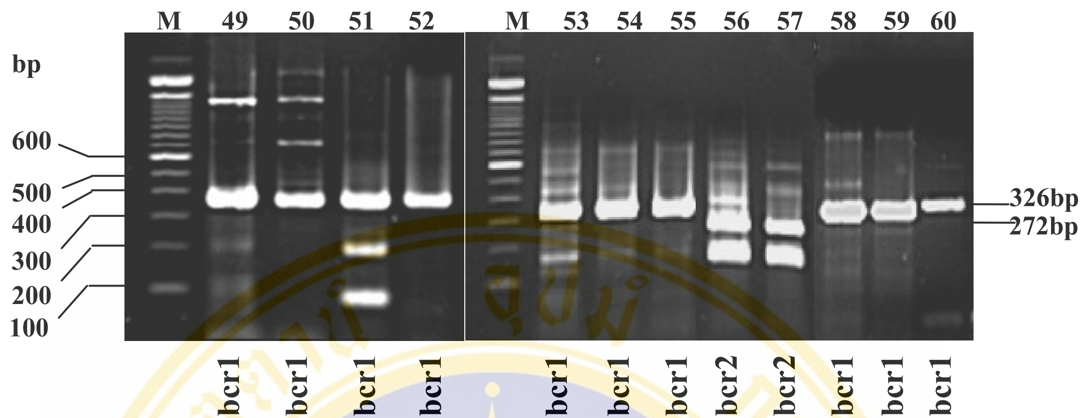

Figure 24 Agarose gel electrophoresis of PCR products from 57 cases with bcr1 patients, 2 samples of NB4 cell line and 3 cases of bcr2 pateints. Electrophoresis was performed on 2% agarose gel in 0.5X TBE buffer. Size of PCR products of bcr1 were 326 bp and bcr2 were 272 bp. Lane M is 100 bp DNA ladder used as a standard-size marker. PCR products and markers were visualized by ethidium bromide staining.

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5. Immunophenotypic characteristics of APL and non-APL cases

The flow cytometric analysis of AML with t(8;21)(q22;q22) APL with t(15;17)(q22;q21), and AML with abnormal bone marrow eosinophils and inv(16)(p13q22) were shown in Figure 25, A, B, C, respectively. Normal BM was shown for comparison in Figure 25, D. The patterns of SSC and intensity of CD45 expression were used to characteristic each subtype.

The immunophenotypes of AML patients demonstrated high expression of myelocytic antigens, including CD33, CD13, and myeloperoxidase (MPO). Antigens that were frequently expressed in AML except APL were CD34 and HLA-DR. CD14 was expressed highly in AML with inv(16). Lymphoid antigens were always negative in all types, except CD19 that was formed in 48% of AML with t(8;21) (Table 13).

The immunophenotypic data of AML patients with specific karyotypes are summaried in Table 13. The expression of CD13, CD33, MPO, CD117, CD14, CD19, CD56 and CD34 of APL with t(15;17) was observed in 15/15 (100%), 16/16 (100%), 13/14 (92.9%), 6/10(60.0%), 1/16 (6.3%), 5/16 (31.3%), 1/16 (6.3%) and 3/16 (18.2%), respectively.

None of HLA-DR, GlyA, CD3, CD5, CD7, CD10 and CD20 was expressed in APL with t(15;17) patients. Expression of CD2, CD14 and CD56 were seen in 2/9 (22.2%), 1/16 (6.3%) and 1/16 (6.3%) of APL patients with t(15;17), respectively. CD19 was observed in 5/16 (31.3%).

Patients with t(8;21) strongly expressed CD13, CD33, MPO, CD34, CD117, CD11c, CD56 and HLA-DR, whereas inv(16) highly expressed CD13, CD33, MPO, CD14, CD11c, CD117 and HLA-DR. For 11q23 patients, the strongly expression of CD33, CD11c, CD13, CD117 and HLA-DR were found.

The percentage of mean expression of CD34, HLA-DR and CD11c of AML with t(15;17) lower than another WHO subgroup (P-value<0.01). CD56 and CD19 highly express in AML with t(8;21) (P-value<0.01). CD14 express in AML with inv(16) higher than another group (P-value<0.01).

Morphologic analysis of each type of AML was also shown for comparison. Patients with t(15;17) had predominantly promyelocytic cells as expected.

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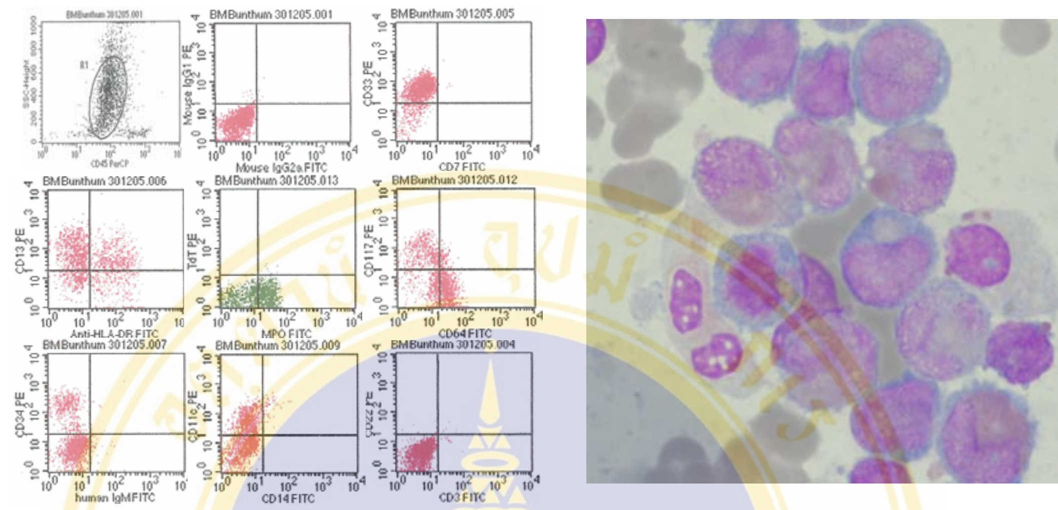
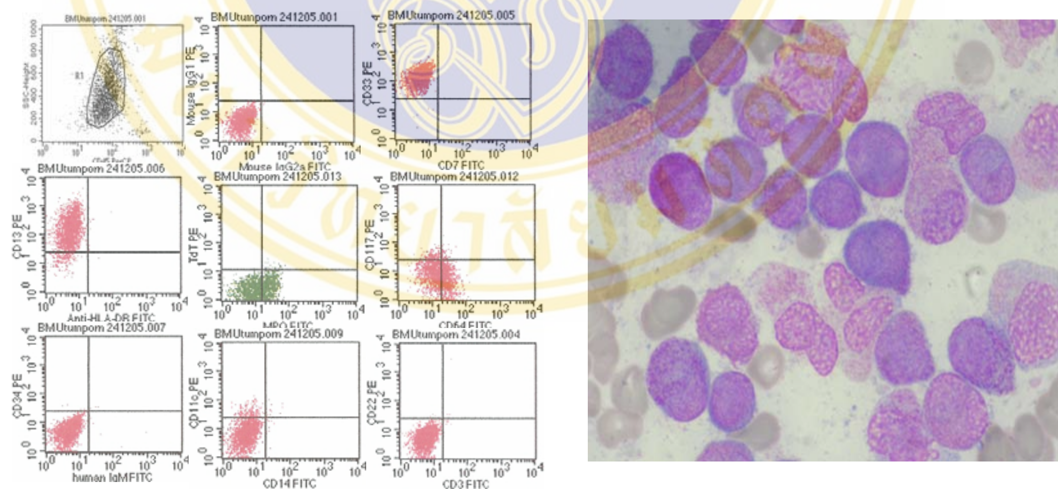
A. Acute myeloid leukemia with t(8;21)(q22;q22)



B. Acute promyelocytic leukemia with t(15;17)(q22;q21)



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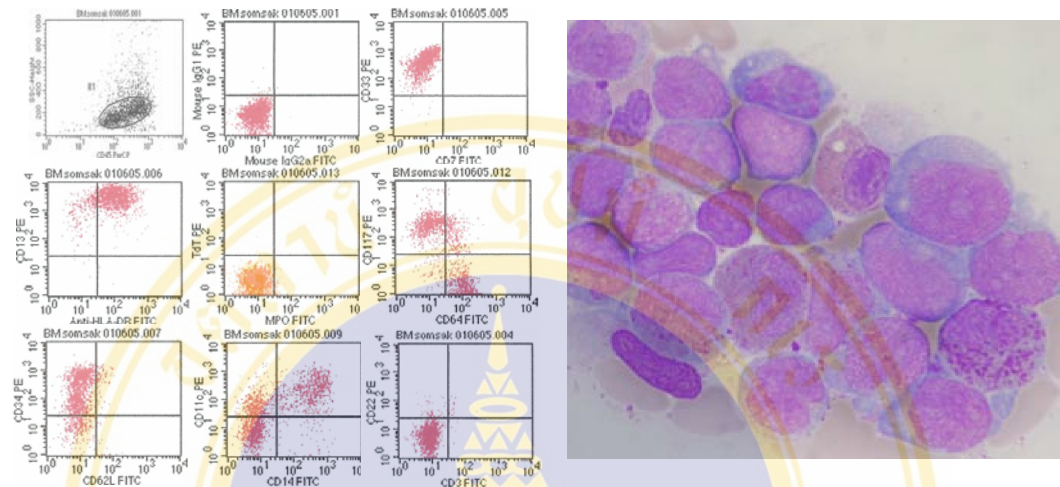
C. Acute myeloid leukemia with abnormal bone marrow eosinophils and inv(16) (p13q22)



D. Normal marrow

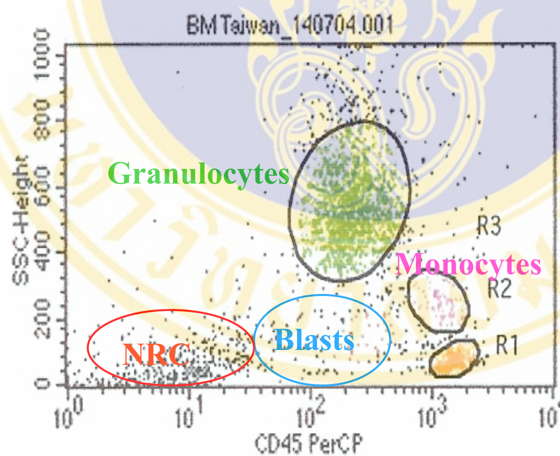


Figure 25 Immunophenotypic analysis of normal and leukemic bone marrow by CD45-side scatter analysis and their corresponding morphology. (A) AML with t(8;21)(q22;q22) (B) APL with t(15;17)(q22;q21) (C) AML with abnormal bone marrow eosinophils and inv(16) (p13q22). These patterns are representative and are not specifically diagnostic in the absence of other data.

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Table 13 Immunophenotypic patterns of AML patients by WHO classification.

Antigen	t(15;17) (n=16)			t(8;21) (n=50)			inv(16)(p13q22) (n=11)			11q23 (n=8)		
	Pos./Ex.	%pos.	Mean expression	Pos./Ex.	%pos.	Mean expression	Pos./Ex.	%pos.	Mean expression	Pos./Ex.	%pos.	Mean expression
CD34+	3/16	18.8	8.4	41/50	82.0	45.3	10/11	90.9	50.9	7/8	87.5	71.1
HLA-DR+	0/16	0.00	2.9	44/50	88.0	63.0	11/11	100	78.3	8/8	100	91.6
CD34+,HLA-DR+	0/16	0.00		40/50	80.0		10/11	90.9		7/8	87.5	
CD34-,HLA-DR+	0/16	0.00		4/50	8.0		1/11	9.1		1/8	12.5	
CD34+,HLA-DR-	3/16	18.8		1/50	2.0		0/11	0.00		0/8	0.00	
CD34-,HLA-DR-	13/16	81.8		5/50	10.0		0/11	0.00		0/8	0.00	
CD13	15/15	100	84.7	47/49	95.9	69.4	11/11	100	95.5	7/8	87.5	71.9
CD33	16/16	100	96.4	47/49	95.9	73.9	11/11	100	87.7	8/8	100	98.4
CD11c	1/15	6.7	11.1	33/37	89.2	47.3	10/10	100	58.0	7/7	100	91.8
CD14	1/16	6.3	3.1	3/50	6.0	8.6	9/11	81.8	39.3	2/8	25.0	17.9
CD117	6/10	60.0	36.8	14/18	77.7	41.8	5/6	83.3	39.3	4/5	80.0	53.9
MPO	13/14	92.9	73.3	34/37	91.9	58.8	10/10	100	37.1	6/7	85.7	10.8
GlyA	0/16	0.0	1.5	0/49	0.0	2.4	0/11	0.0	1.9	0/8	0.0	1.7
CD56	1/16	6.3	6.1	38/45	84.4	54.6	0/11	0.0	5.2	2/8	25.0	34.6
CD10	0/16	0.0	0.2	0/45	0.00	0.4	0/9	0.0	0.2	0/8	0.0	0.4
CD19	5/16	31.3	25.1	24/50	48.0	29.2	0/10	0.0	4.7	1/8	12.5	14.7
CD20	0/16	0.00	0.3	0/48	0.00	0.8	0/11	0.0	0.9	0/8	0.0	0.4
CD2	2/9	22.2	7.2	0/33	0.00	1.3	0/8	0.0	4.7	0/4	0.0	0.9
CD3	0/16	0.0	2.8	1/50	2.0	2.3	0/11	0.0	2.6	0/8	0.0	3.8
CD5	0/16	0.0	0.4	0/49	0.00	1.1	0/11	0.0	2.7	1/8	12.5	5.3
CD7	0/16	0.0	0.5	4/50	8.0	3.5	0/11	0.0	1.2	1/8	12.5	8.0
TdT	0/16	0.0	4.2	1/37	2.7	3.0	0/9	0.0	1.8	0/7	0.0	1.5

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The positive predictive values (PPV) and negative predictive values (NPV) of the features associated with *PML/RARα* were shown in Table 14. a $CD34^-$ and $HLA\text{-}DR^-$ had the highest PPV (72.2%) and the highest NPV (100%) for APL. In contrast, $CD34^+$ and $HLA\text{-}DR^+$ had the highest the highest PPV (100%) for t(8;21) AML.

Table 14 Diagnostic values of CD34 and HLA-DR in the differentiation of t(15;17) APL from t(8;21) AML *

Immunophenotype	t(15;17)APL	t(8;21) AML
$CD34^+$ or $HLA\text{-}DR^+$		
Incidence, No.(%)	3/16 (18.8)	45/50 (90.0)
PPV, %	NA	93.8
NPV, %	NA	37.5
$CD34^+$ and $HLA\text{-}DR^+$		
Incidence, No.(%)	0/16 (0)	40/50 (80.0)
PPV, %	NA	100
NPV, %	NA	72.2
$CD34^-$ and $HLA\text{-}DR^-$		
Incidence, No.(%)	13/16 (81.3)	5/50 (10.0)
PPV, %	72.2	NA
NPV, %	100	NA

* PPV indicates positive predictive value; NPV, negative predictive value; NA, not applicable.

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The sensitivity, specificity and predictive values of individual markers associated with *PML/RARα* fusion gene were shown in Table 15.

The PPV of MPO, CD33, CD13 and CD117 were 72.3%, 46.7%, 75.8% and 93.8% respectively. CD13 and CD33 had the highest specificity (100%), while CD13 had highest sensitivity (95.9%). MPO also had highly sensitivity (91.9%) with high specificity (92.9%), with the NPV of 25%. CD 117 had the highest PPV (93.8%) and highly NPV (77.2%), although the sensitivity and specificity were low.

Table 15 Comparison of four different markers to assign APL.

Marker	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
CD117	43.8	18.8	93.8	72.3
CD33	77.8	100	46.7	0
CD13	95.9	100	75.8	0
MPO	91.9	92.9	72.3	25.0

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Immunophenotypic and morphologic characteristic of APL patients with or without *PML/RAR*α fusion gene are shown in Figure 26. APL cases strongly expressed MPO, CD13, CD33 and CD117 but did not express CD34 or HLA-DR (Figure 26, A. and Figure 27, A.). The APL morphology with or without *PML/RAR*α showed similar morphologic pattern with predominant promyelocytes in the BM (Figure 26, B and Figure 27, B).

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A. **B.**

Figure 26 Immunophenotypic (A) and morphologic (B) characteristics of patients No.895 with t(15;17) karyotype and *PML/RAR*α fusion gene. Arrow shows a promyelocyte.

A. **B.**

Figure 27 Immunophenotype (A.) and morphology (B.) characteristic of patients No.873 with normal karyotype and without *PML/RAR*α fusion gene. Arrow shows a promyelocyte.

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The case of APL was found to has *PML/RAR*α and t(8;21) as shown in Figure 28. This case had typical morphologic and immunophenotypic characteristics of APL with predominant promyelocytic cells and absence of CD34 and HLA-DR.

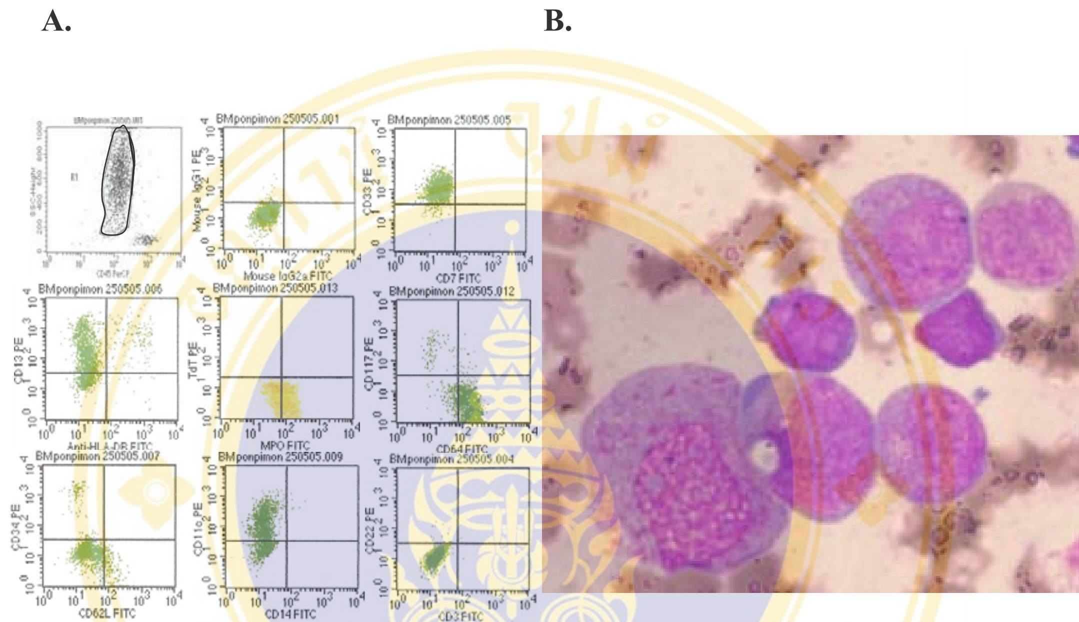

Figure 28 Immunophenotypic and morphologic characteristics of patients No.750 with t(8;21) and with *PML/RAR*α fusion gene

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The case of APL was found to has *PML/RAR*α with normal karyotype as shown in Figuer 29. This case had typical morphologic and immunophenotypic characteristics of APL with predominant promyelocytic cells and absence of CD34 and HLA-DR.

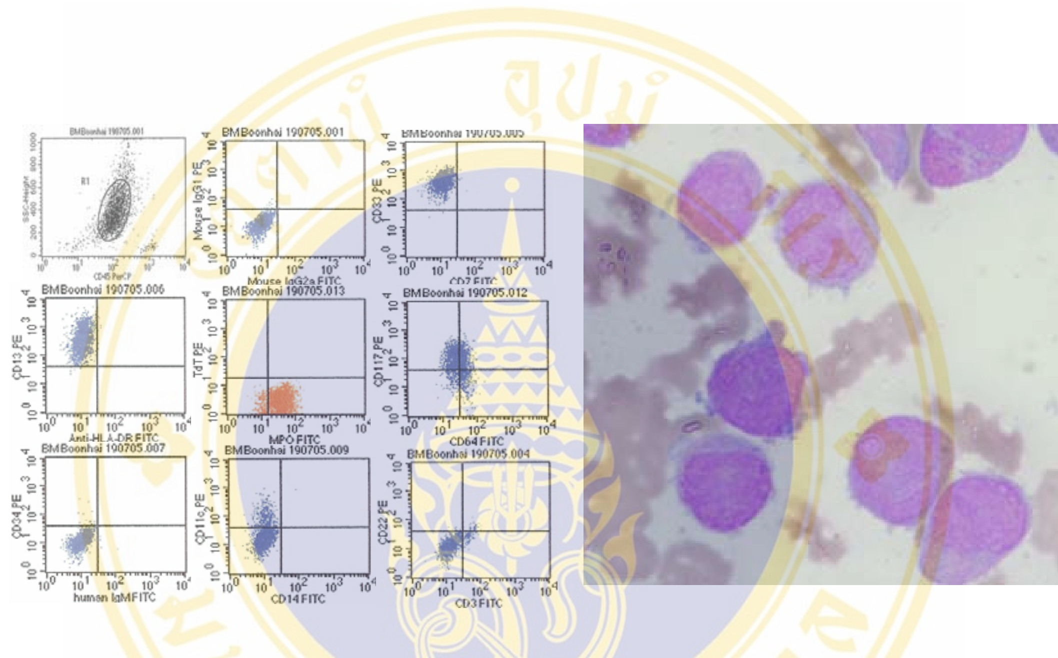

Figure 29 Immunophenotypic and morphologic characteristics of patients No.791 who had a normal karyotype but was found to carry *PML/RAR*α fusion gene.

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6. Immunophenotypic characteristic of different *PML/RARα* gene variant

The Immunophenotypic patterns of APL patients with different *PML/RARα* variants are shown in Table 16. The expression of myeloid markers such as CD13, CD33 and MPO are highest in all isoforms, whereas lymphoid markers were absence. The most important markers CD34 and HLA-DR consistenly were absent in bcr2 and bcr3 (100%), and bcr1 (91.2%).

Table 16 Immunophenotypic Patterns of APL Patients with *PML/RARα* variants

Markers	***PML/RARα* variants**						P-value*
	bcr1 (n=57)		**bcr2 (n=3)**		**bcr3 (n=2)**		
	Pos./Ex.	**%**	**Pos./Ex.**	**%**	**Pos./Ex.**	**%**	
CD34+[a]	5/57	8.7	0/3	0.0	0/2	0.0	NS
HLA-DR+	0/57	0.00	0/3	0.0	0/2	0.0	NS
CD34+,HLA-DR+	0/57	0.00	0/3	0.0	0/2	0.0	NS
CD34-,HLA-DR+	0/57	0.00	0/0	0.0	0/2	0.0	NS
CD34+,HLA-DR-	5/57	8.8	0/3	0.0	0/2	0.0	NS
CD34-,HLA-DR-	52/57	91.2	3/3	100	2/2	100	NS
CD13	55/57	96.5	2/2	100	2/2	100	NS
CD33	54/56	96.4	3/3	100	2/2	100	NS
CD11c	24/33	72.7	1/3	33.3	1/2	50.0	NS
CD14	0/57	0.00	0/3	0.0	0/2	0.0	NS
CD117	17/21	81.0	2/3	66.6	1/2	50.0	NS
MPO	37/40	92.5	3/3	100	2/2	100	NS
GlyA	0/57	0.00	0/3	0.0	0/2	0.0	NS
CD56	20/57	35.1	0/3	0.0	0/2	0.0	NS
CD10	0/54	0.00	0/3	0.0	0/2	0.0	NS
CD19	15/57	26.3	1/3	33.3	0/2	0.0	NS
CD20	0/55	0.00	0/3	0.00	0/2	0.0	NS
CD2	2/37	5.4	1/1	100	ND	ND	NS
CD3	1/57	1.2	0/3	0.0	0/2	0.0	NS
CD5	0/56	0.00	0/3	0.0	0/2	0.0	NS
CD7	3/57	5.3	0/3	0.0	0/2	0.0	NS
TdT	0/40	0.0	0/3	0.0	0/2	0.0	NS

*Chi square test, NS; not significant, ND; not done

[a] Mean expression of CD34 was 41.9%.

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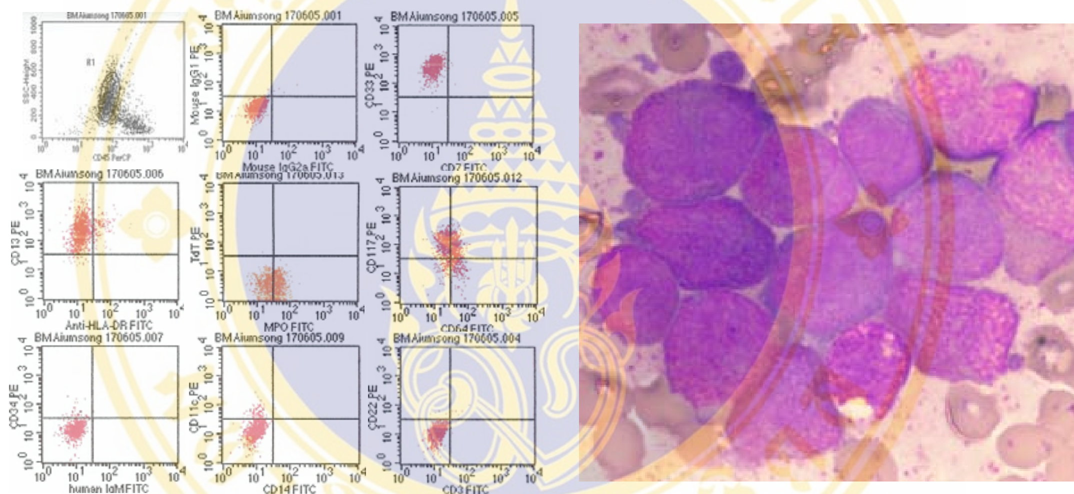
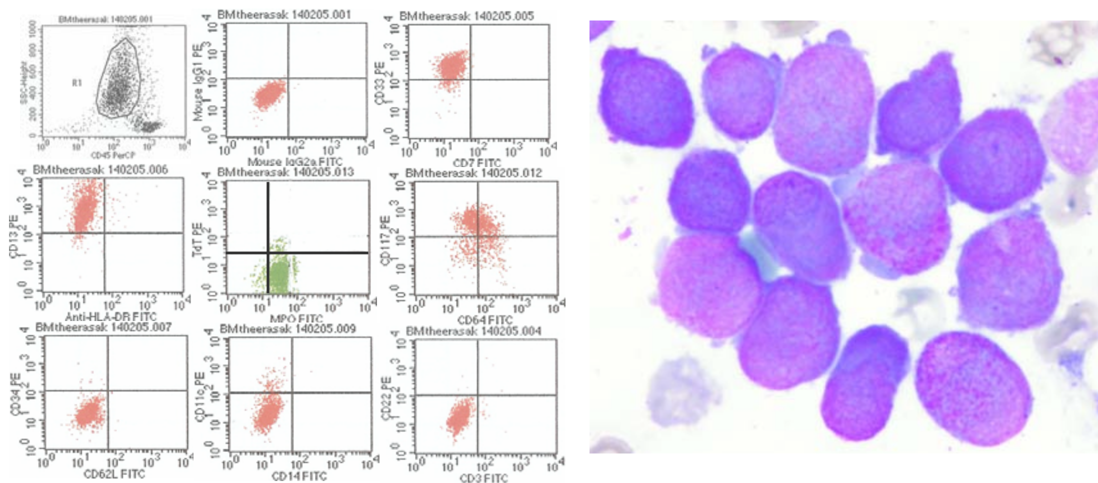
The Immunophenotypic and morphologic characteristics of APL patients with bcr1, bcr2 and bcr3 were shown in Figure 30, A, B and C, respectively. The pattern of immunophenotypes does not different and the morphology shows typical promyelocytes.

A. Immunophenotypic and morphologic characteristics of patients with bcr1 *PML/RARα* fusion gene



B. Immunophenotypic and morphologic characteristics of patients with bcr2 *PML/RARα* fusion gene



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C. Immunophenotypic and morphologic characteristics of patients with bcr3 *PML/RAR*α fusion gene

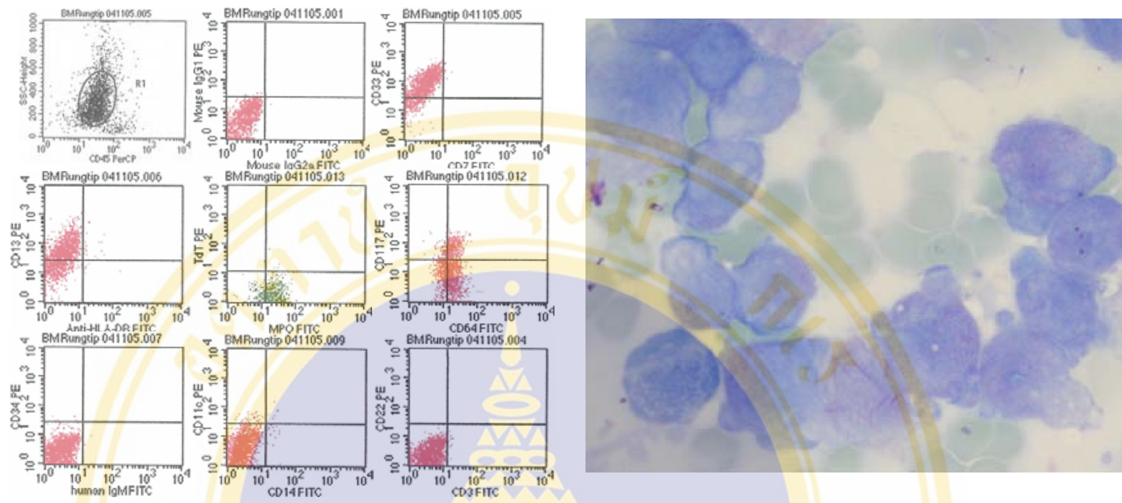

Figure 30 Immunophenotypic and morphologic characteristics of patients with *PML/RAR*α fusion gene variants (A) patients No.766 with bcr1, (B) patients No 670. with bcr2 and patients No.872 with bcr3.

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7. Clinical characterization of Thai APL patients with or without *PML/RARα* fusion gene

To determine if clinical differences between APL patients with and without *PML/RARα* fusion gene exist, the patients' clinical characteristics at the initial diagnosis were compared as shown in Table 17. The presence of any type of *PML/RARα* fusion gene was markedly associated with low leukocyte counts and platelet (Plt) count as compared to the negative cases ($p<0.05$). No association was observed between the *PML/RARα* fusion gene and negative group in term of hemoglobin (Hb). t(15;17) was frequently found in young patients (28 years old). With respect to bcr variants, the bcr2 patients were relatively younger than other type (29 versus 41 and 55). However, no statistical significance could be demonstrated in any parameter (Table 18).

Table 17 Demographic data of 80 Thai APL patients with t(15;17) and *PML/RARα*, Thai APL patients present *PML/RARα* fusion gene, but without t(15;17) karyotype and APL absence both t(15;17) and *PML/RARα* fusion gene.

Parameter	Presence of both t(15;17) and *PML/RARα* (16 cases)	Absence of t(15;17) with presence of *PML/RARα* (46 cases)	Absence of both t(15;17) and *PML/RARα* (18 cases)*	P value
Median age (year)	28	47.5	48	0.002
Age range (year)	15-47	15-84	16-78	
Age >60 yrs (%)	0	30.4	27.7	
Female:Male ratio	9:7	29:17	14:4	0.390
Median WBC ($x10^9/L$)	5.2	38.8	21.7	0.017
% of patients with WBC count <20 $x10^9/L$	75.0	21.8	44.5	
% of patients with WBC count 20-50 $x10^9/L$	18.8	28.2	22.2	
% of patients with WBC count >50 $x10^9/L$	6.3	50.0	33.3	
Median Hb (g/dL)	7.5	8.1	8.5	0.574
Median Plt ($x10^9/L$)	15.5	46.0	30.0	0.003

* Normal karyotype 9 cases, trisomy 8 2 cases, t(8;21) 1 case, t(8;16) 1 case and no metaphase (chromosome analysis could not be done) 5 cases

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Table 18 Demographic data of APL patients with difference *PML/RAR*α variants.

Parameter	bcr1 (57 cases)	bcr2 (3 cases)	bcr3 (2 cases)	total (62 cases)
Median age (year)	41	29	55	41
Age range (year)	15-84	17-42	27-83	15-84
Age>60 yrs (%)	24.5	0	50.0	24.2
Female:Male ratio	35:22	1:2	2:0	38:24
Median WBC ($x10^9$/L)	26.4	45.9	23.5	28.2
% of patients with WBC count <20 $x10^9$/L	31.5	33.3	50.0	45.2
% of patients with WBC count 20-50 $x10^9$/L	30.0	33.3	50.0	24.2
% of patients with WBC count >50 $x10^9$/L	38.5	33.3	0.0	29.0
Median Hb (g/dL)	8.1	5.9	4.7	7.9
Median Plt ($x10^9$/L)	40.0	41.0	20.5	38

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8. Incidence of *FLT3* mutation in Thai adult APL patients

A total of 80 samples were screened for the presence of *FLT3* mutations (Table 19). An ITD was found in 21 of total 80 APL cases (26.3%). A mutation in the TKD was present in 10 of these APL cases (12.5%). 49 patients (61.2%) did not have *FLT3* mutations.

FLT3 mutation also found in AML with t(8;21), 6.1% had ITD, and ITD and TKD mutation as shown in Figure 20. AML with inv(16) did not have *FLT3* mutation.

Table 19 Frequency of FLT3 mutations in 80 Thai adult APL patients.

Type of mutation	**APL (80 cases)**	
	N	**%**
ITD	21	26.3
TKD	10	12.5
No ITD &TKD	49	61.2

Table 20 Incidence of *FLT3* mutation in Thai adult AML with t(8;21) and AML with inv(16) patients.*

Type of mutation	**AML with t(8;21) (33 cases)**		**AML with inv(16) (6 cases)**	
	N	**%**	**N**	**%**
ITD	2	6.1	0	0
TKD	1	3.0	0	0
ITD &TKD	2	6.1	0	0
No ITD &TKD	28	84.8	6	100

*This *FLT3* mutation data come from the study of Miss Amporn Leecharendkeat and Mr. Narongrit Srithana.

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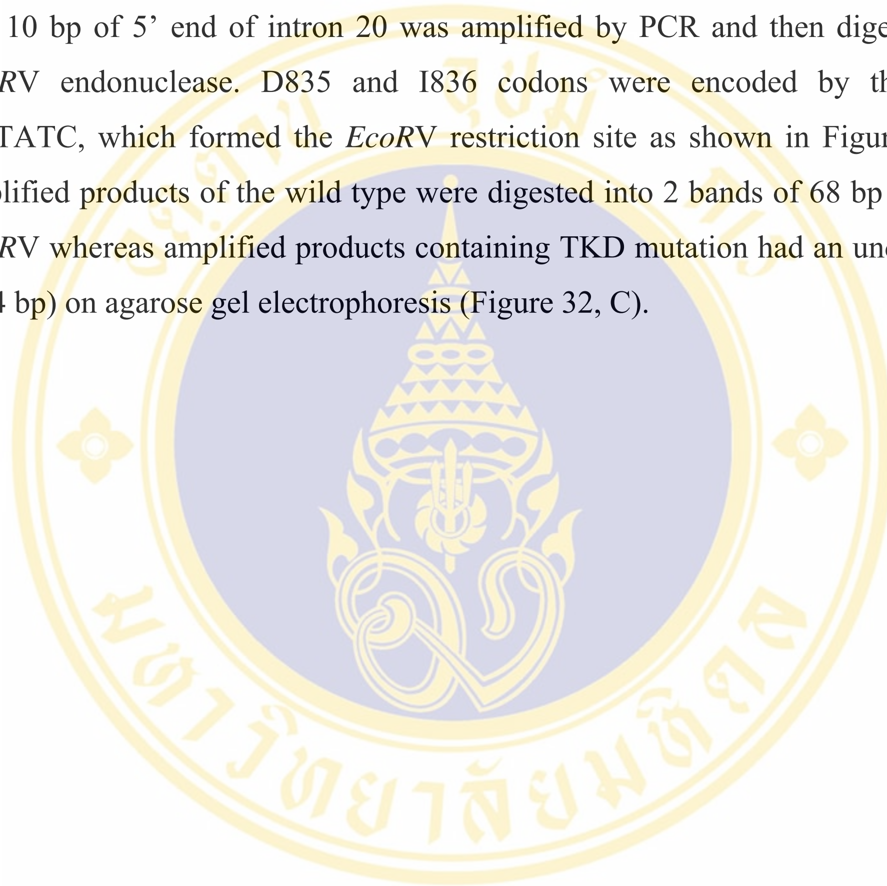
Figure 31 showed the analysis of *FLT3-ITD* by PCR. The PCR products with wild-type *FLT3* was 329 bp. The *FLT3*-ITD amplification yielded a higher molecular weight product on a 2% agarose gel stained with ethidium bromide.

The alteration in exon 20 of *FLT3* gene was studied using the technique as shown in Figure 32. A 114-bp fragment comprised 104 bp of the 3’ end of exon 20 and 10 bp of 5’ end of intron 20 was amplified by PCR and then digested with the *EcoR*V endonuclease. D835 and I836 codons were encoded by the nucleotide GATATC, which formed the *EcoR*V restriction site as shown in Figure 32, B. The amplified products of the wild type were digested into 2 bands of 68 bp and 46 bp by *EcoR*V whereas amplified products containing TKD mutation had an undigested band (114 bp) on agarose gel electrophoresis (Figure 32, C).



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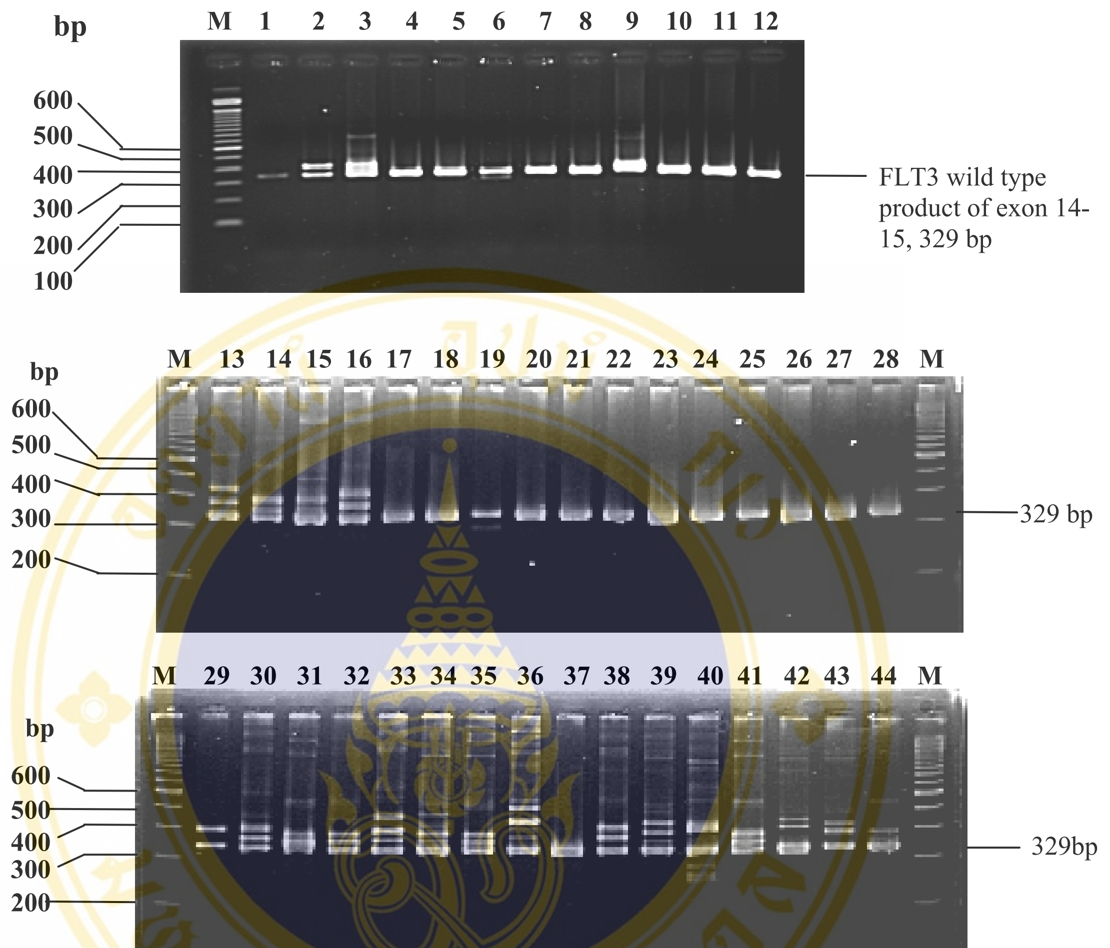

Figure 31 Agarose gel electrophoresis of PCR products from 21 cases with *FLT3*-ITD mutation. Electrophoresis was performed on 2% agarose gel in 0.5X TBE buffer. The size of PCR product with wild type *FLT3* was 329 bp and aberrant ITD products identified as the higher molecular weight bands than the wild type. Lane M is a 100 bp DNA ladder used as a standard-size marker. PCR products and markers were visualized by ethidium bromide staining.

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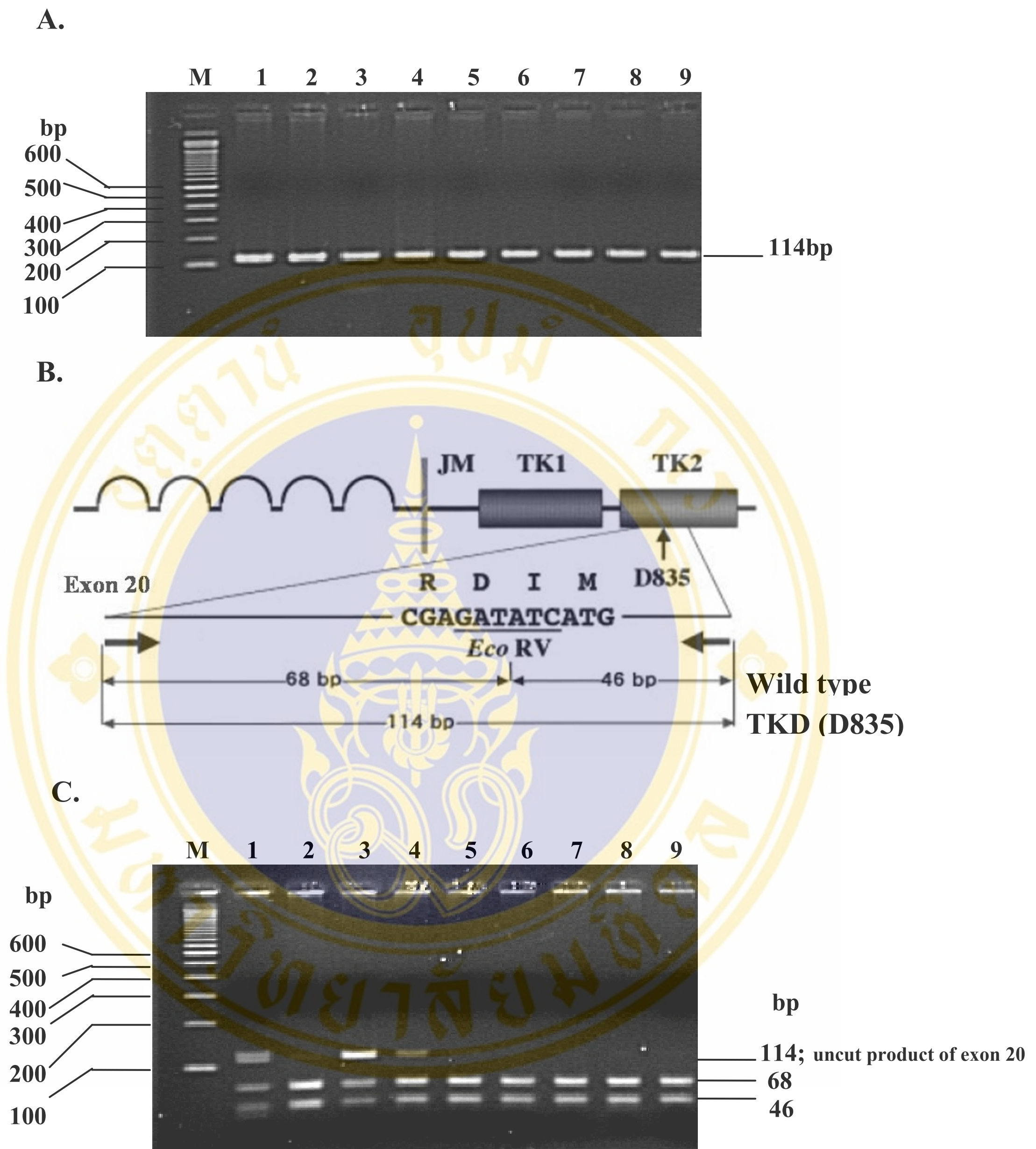

Figure 32 Detection of TKD mutation in the *FLT3* gene. To detect TKD mutations, exon 20 was amplified by PCR (A), and then digested with *EcoR*V endonuclease (Yamamoto Y. et al, 2001) (B). The amplified products of the wild-type were digested into 2 bands (68 bp and 46 bp) by EcoRV whereas the amplified products containing TKD mutation had an undigested band (114 bp) (C, lanes 1, 3 and 4). Lane M is a 100 bp DNA ladder used as a standard-size marker. PCR products and markers were visualized by ethidium bromide staining.

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The calculation of *FLT3* and *PML/RARα* variant is shown in Table 21. An ITD was found in 15 cases (18.8%) of bcr1 and 1 case (1.3%) of bcr2, respectively. The TKD mutation were present in 3 cases (3.7 %) of bcr1 and none of bcr2, respectively. Both bcr3 cases had *FLT3*-TKD mutation.

Table 21 Incidence of and *FLT3* mutations in APL patients with *PML/RARα* fusion gene

Type of mutation	***PML/RARα* fusion gene variants**							
	bcr1 (n=57)		**bcr2 (n=3)**		**bcr3 (n=2)**		**Negative(n=18)**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
ITD	15	18.8	1	1.3	0	0.00	5	6.3
TKD	3	3.7	0	0.00	2	2.5	5	6.3
Total FLT3	18	22.5	1	1.3	2	2.5	10	12.6

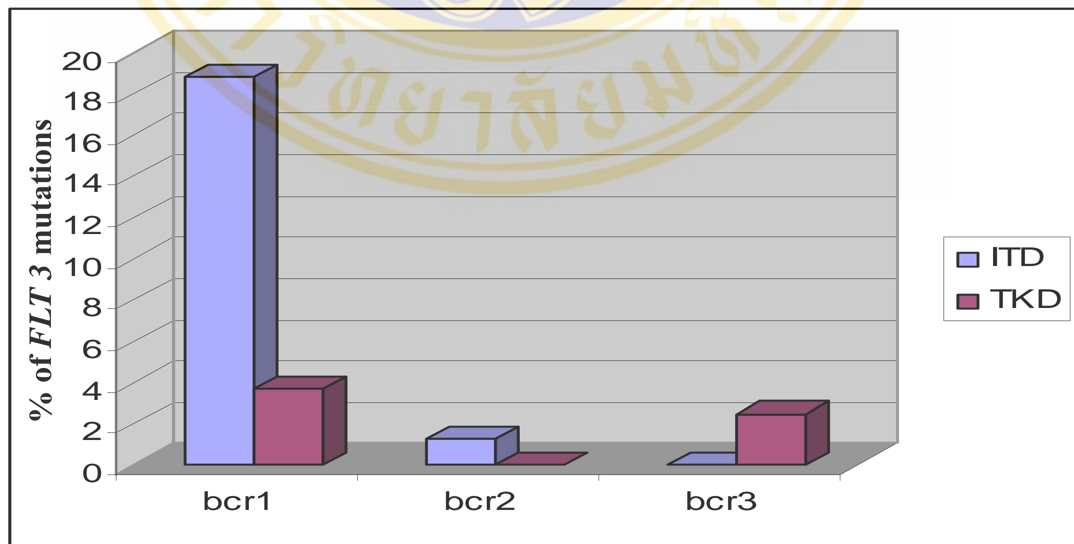

Figure 33 Type of *FLT3* mutations in *PML/RARα* positive APL (n=62)

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CHAPTER VI
DISCUSSION

In this study, I evaluated the frequency and type of *PML/RARα* fusion gene variants in 80 newly diagnosed Thai adult APL patients. The most common *PML/RARα* fusion gene isoform in Thailand was the bcr1 (L type). The proportion of bcr1 isoform of 92% among Thai APL patients was higher than that of other countries (Table 22). In the European group, the incidence of bcr1 was 45-67.5% followed by bcr3 (26.5-45%) and bcr2 (5-10%) (84-90). Three American studies showed variable frequencies of *PML/RARα* isoforms, with the incidence of bcr1 of 45-70%, bcr2 of 7-10% and bcr3 of 20-45%, respectively (28, 41 91). The incidence of *PML/RARα* fusion gene in Asian countries was previously known only for APL patients in Japan and China. In the Japanese study, the combined frequency of bcr1 and bcr2 was 70% with bcr3 of 30% (93). One Chinese study reported the incidence of bcr1, bcr2 and bcr3 of 64%, 7% and 29%, respectively (51). Similarly, another Chinese study reported the incidence of bcr1, bcr2 and bcr3 of 66.7%, 6% and 27.3%, respectively (94). The bcr2 isoform was the least frequently found isoform in most studies. In two studies, bcr3 was more prevalent than other countries (45%) (41, 84). In our cohort, surprisingly, bcr2 and bcr3 was very rare with only 3 cases and 2 cases detected, respectively.

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Table 22 The distribution of *PML/RAR*α fusion gene isoforms in APL patients from various countries.

Reference		Country	% bcr1 (n)	% bcr2 (n)	% bcr3 (n)	Total number studied
Grignani F et al. (84)	Europe	Italy	45	10	45	N/A (Review article)
Lo-Coco F et al.(85)	Europe	Italy	55	8	35	N/A (Review article)
Guglielmi C et al(86)	Europe	Italy	58.5 (93)		41.5(66)	196
van Dongen JJ et al. (87)	Europe	Europe	55	5	40	N/A (Review article)
Chillon CM et al.(88)	Europe	Spain	67.5 (23)	6 (2)	26.5 (9)	34
Burnett AK et al.(89)	Europe	UK	61 (114)		39 (72)	186
Sucic M et al.(90)	Europe	Croatia	62 (8)		38 (3)	13
Slack JL et al. (91)	America	USA	57 (32)	7 (4)	36 (20)	56
Melnick A and Licht JD(28)	America	USA	70	10	20	N/A (Review article)
Ruggero D. Et al (41)	America	USA	45	10	45	N/A (Review article)
Douer D et al.(92)	America	Latin America	75 (39)	10 (5)	15 (8)	52
Naoe T et al.(93)	Asia	Japan	70 (84)		30 (36)	120
Geng JP. et al.(51)	Asia	China	64 (18)	7 (2)	29 (8)	28
Geng JP. et al.(94)	Asia	China	66.7 (22)	6.0 (2)	27.3 (9)	33
This study	**Asia**	**Thailand**	**92 (57)**	**5 (3)**	**3 (2)**	**62**

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The age at onset of AML is 60 years in the western series (95). However, Thai AML patients were known to be much younger (median age of 40 years) as reported by Auewarakul et al (96). The age at onset of APL patients in the west was 40 which was younger than non-APL patients (10). Guglielmi et al (86) reported the median age of 196 APL patients of 40 years, with equal distribution of two sexes. In this study, the median age of our patients was 40 (range 15-84) but we had predominantly females in our Thai APL cases. It is unclear why females were affected more than males in Thailand.

The impact of *PML/RARα* isoforms on the clinical features and outcome has been reported (40). In particular, the bcr3 isoform had been consistently associated at presentation with hyperleucocytosis as well as with microgranular morphology. Sucic et al also found that patients with bcr3 had the short median first remission and overall survival in comparison with patients with bcr1 (90). In this study, the median WBC counts of patients with bcr3 was not different from other isoforms, although it is difficult to conclude due to the low incidence of bcr3 in our population (n=3). The morphology of the bcr3 cases in this study was also not microgranular.

I also set out to determine the specific immunophenotypes that could be used to predict t(15;l7) and *PML/RARα* isoforms. The immunophenotypes of AML patients with recurrent translocations according to the recent WHO classification, i.e., AML with t(8;21)(q22;q22), APL with t(15;17)(q22;q21), and AML with abnormal bone marrow eosinophils and inv(16) (p13q22) and AML with 11q23 were compared. The high expression of CD13, CD33, MPO, and CD117 and lack of lymphoid antigen such as CD2, CD3, CD5, CD7, CD10 in patients with t(8;21) were similar to APL with t(15;17). CD 19 and CD56 were highly expressed in AML with t(8;21) as compared to APL ($p=0.011$ and $p<0.001$, respectively). CD14 was mostly found in AML with inv(16) as contrast to APL with *PML/RARα* ($p<0.001$). Patients with 11q23 abnormalities had high expression of CD33, CD13, HLA-DR and CD34 and most cases were classified as FAB M2 (42.9%) and M4 (28.6%). Other types of translocations except t(15;17) had high HLA-DR and CD34 expression. HLA-DR negative and CD34 negative were thus helpful in predicting APL with t(15;17) or

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PML/RARα fusion gene as both markers when negative had the highest PPV in APL. Although CD34 has been shown to be associated with micro/hypogranular morphology, expression of CD2 and bcr3 isoform, in this study, I did not find such correlation. The aberrant lymphoid antigen frequently found in Thai APL with *PML/RARα* was CD2 which was significantly different from other types of AML in this series (p=0.007). Our patients with bcr isoforms had the same pattern in immunophenotypic analysis.

The frequency of *FLT3*-ITD in western APL patients as reported from Gilliland et al (72) was approximately 35%, and correlated with the overall percentage of APL patients who had a poor prognosis. Gale et al (97) also studied 203 patients with *PML/RARα* positive APL and found 43% of the patients had *FLT3* mutations with 32.19% (65 cases) ITD, 9.35% D835/I836, and 1.97% had ITD and D835/I836. In my study, *FLT3* mutation was found in 38.8% of APL patients. *FLT3*-ITD was most common in Thai patients with bcr1 isoform, while bcr 3 was strongly associated with *FLT3*-TKD (100% in bcr3 cases). Kottaridis et al (73), found that *FLT3*-ITD was associated with M3v, hyperleucocytosis and bcr3 isoform. In this study, the outcome of our APL patients with or without *FLT3* was not different (p=0.472).

The current concept of carcinogenesis is based on the model of colorectal cancer whereby multiple "hits" are required to initiate and propagate the cancerous lesion from benign epithelium. In vitro, the cooperation of at least two genes such as *RAS* and *MYC* was also demonstrated using transfection assays (98). In the past, the genetic abnormalities in acute leukemia were thought to be non-specific as various structural and numerical chromosome abnormalities were randomly found. However, with the cloning of novel transcription factor and receptor tyrosine kinase genes at the chromosome breakpoints as well as the discovery of mutations of gene whose normal functions are essential in the cell proliferation and differentiation processes, the concept of multi-step leukemogenesis was recently realized (9). In this study, the data supports the concept of cooperative mutations in a subgroup of APL patients as discussed in the previous section. The primary genetic event in APL, i.e., the *PML/RARα* impairs promyelocye differentiation whereby another 'hit', i.e., the *FLT3*

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mutation promotes proliferation and survival of leukemic cells. Similarly, in AML with t(8;21), *AML1/ETO* may be the primary genetic event that impair differentiation, whereas c-Kit receptor tyosone kinase has been shown to be a possible second hit. It should be explored further what the underlying genetic events in the Thai APL patients without detectable *FLT3* mutation. Nevertheless, the presence of *FLT3* mutation in APL patients with *PML/RAR*α would be valuable for the future design and application of molecular tageting therapy against both genetic abnormalities in Thai population.

Interestingly, I also found a coexistence of t(8;21) in two APL patients. Bonomi et al(99), also reported one case of APL with t (15;17) and *PML/RAR*α fusion gene who relapsed with a typical FAB M2 morphologic features and the karyotype was 45,XY,t(8;21). Lima et al (100) also reported the simultaneous expression of *PML/RAR*α and *AML1/ETO* fusion genes by RT-PCR in the same patient. Two reports also published the results of FISH analysis that detected both t(8;21) and t(15;17) in M2 patients (101, 102). It is unclear what mechanisms are involved in such cases.

The cytogenetic analysis has long been very important in the diagnostic steps of APL. However, it takes a longer time to yield the results (3-7 days). Moreover, in this study, t(15;17) by karyotyping was detected in only 20% of APL patients diagnosed by morphology and flow cytometry whereas molecular analysis was much more sensitive (77.5% of 80 cases) and takes less time to yield the result (24 hours). However, cytogenetic analysis still has the advantage of identifying other structural and numerical abnormalities involving other chromosomes. Overall, Thai APL patients had a lower incidence of t(15;17) and/or *PML/RAR*α (77.5%) than that of the west with the incidence of 98% (40). The true incidence of t(15;17) by karyotyping in Thai APL patients could be higher than 20% as FISH was not performed in this study due to its high cost and lack of experienced cytogenetists at the time of the study. If FISH were performed in combination with a nested RT-PCR, the overall incidence of t(15;17) and/or *PML/RAR*α in this study may be increased. As seen in the results, all cases with t(15;17) and/or *PML/RAR*α had the unique pattern of immunophenotypes.

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With the findings that the immunophenotypic pattern of APL patients with t(15;17) and/or *PML/RAR*α was very unique with a high PPV for CD34-HLA-DR-, flow cytometry should thus be performed first to predict the presence of t(15;17) and/or *PML/RAR*α. Therefore, for practical purposes, the rapid prediction of APL by flow cytometry should be the most useful method for the patients and physicians who need immediate results to justify the initiation of ATRA therapy.

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CHAPTER VII
CONCLUSION

In this study, I developed a rapid molecular assay to determine the incidence and type of *PML/RARα* fusion gene variants in a large cohort of Thai adult APL cases. The finding that bcr1 was the most common variant in the Thai APL patients is in agreement with the results reported from other countries although the bcr2 and bcr3 subtypes appeared to be much rarer in our population.

The unique membrane markers were identified in this study to predict the presence of *PML/RARα* fusion gene variants. The combined use of CD34 and HLA-DR is much more helpful in distinguishing APL from non-APL than either of these antigens alone. None of the APL cases with *PML/RARα* gene was positive for both CD34 and HLA-DR. No statistical differences in hematologic parameters or immunophenotypes were found among APL cases with different bcr subtypes.

FLT3 mutations were predominantly found in APL cases as compared to other AML cases with recurrent and favorable chromosome translocations. *FLT3*-ITD was most commonly found in the bcr1 subtype while *FLT3*-TKD was found in all cases of bcr3. The coexistence of *FLT3* mutation in APL with *PML/RARα* supports the model of multi-step leukemogenesis in a subset of Thai APL patients.

It is hoped that the results of this research would help us not only to understand the molecular pathogenesis of APL but also guide us how to diagnose and predict the *PML/RARα* fusion genes in our leukemia patients by flow cytometry. Moreover, novel therapeutic strategies could also be specifically designed for Thai APL patients using gene-targeting approach against *PML/RARα* and *FLT3* genes. With the ongoing discovery of novel therapeutic targets and development of better drugs for APL, it is not impossible to expect that one day we will be able to call “APL with *PML/RARα* fusion gene” as the most curable disease.

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APPENDIX

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APPENDIX I

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

1. Chemicals

Chemicals	Molecular weight (g/ml)	Source
Absolute ethanol (C_2H_2OH)	46.07	E.Merck, Darmstadtt, F.A., Germany
Agarose ($(C_{12}H_{18}O_9)_n$)		Sokem, Rockland, 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
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 ($C_{10}H_{12}N_2O_{14}P_3Na_4$)	570.1	Promega, Madison, USA.
Ethidium bromide	394.3	Research Organics, Cleaveland, USA.
Ethylenediamine tetra acetic acid tetrasodium salt or EDTA ($C_{10}H_{12}N_2Na_2O_8$)	380.2	E.Merck, Darmstadtt, F.A., Geramany.

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Ficoll (Type 400)	400,000	Sigma chemical, St, Louis, USA
Hydrochloric acid (HCl)	36.50	E.Merck, Darmstadtt, F.A., Germany
Isoamyl alcohol	88.15	E.Merck, Darmstadtt, F.A., Germany
Isopropanol ($CH_3CHOHCH_3$)	60.10	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
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 citrate ($C_6H_5Na_3O_7$)	258.07	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

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2. Instruments

Gel documentation system, UVItec, Cambridge, UK.

Thermal cycler, PCR Sprint Thermal Cycler, CA, USA.

Mini gel electrophoresis apparatus, Labnet international Inc., Edision, USA.

UV-transilluminator, E143, 200-400 V, CONSORT, Turnhout, Hartland, USA.

UV-visible spectrophotometer, Shimadzu UV-160A, Kyoto, Japan.

3. Enzyme

Immolase *Taq* DNA polymerase, Bioline, Germany.

EcoRV, Promega, Madison, USA.

4. DNA markers

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

*Hae*III digested Φx174 DNA, Promega, Madison, USA.

5. Reagents

5.1 10x Phosphate buffer 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 sterile by autoclaving for 15 minutes at 121 °C, 15 lb/square inches and stored at room temperature. It was diluted to 1x before use.

5.2 70% Ethanol

Absolute ethanol	70	ml
Sterile distilled water to	100	ml

The solution was mixed and stored at -20 °C

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5.3 0.5 M EDTA pH 8.0

EDTA	18.60	g

Dissolved in distilled water and adjusted pH 8.0 with NaOH

Added distilled water to	100	ml

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

5.4 5x Tris-borate buffer (TBE buffer)

Tris-base (Sigma)	242	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.

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APPENDIX II

Publications and Presentations

Lists of puplications from this thesis:

1. **Orathai Promsuwicha**, Wanna Thongnoppakhun, Somchai Wongkhantee, Chintana Tocharoentanaphol, Chirayu U Auewarakul. Molecular Characterization of *PML/RAR*α Fusion Gene in Thai patients with Acute Promyelocytic Leukemia (APL) by nested RT-PCR. Thai Journal of Hematology and Transfusion Medicine 2005; 15(4): 247-257.
2. Chirayu U. Auewarakul , Darat Lauhakirti, **Orathai Promsuwicha**, Chanya Munkhetvit. C-kit receptor tyrosine kinase (CD117) expression and its positive predictive value for the diagnosis of Thai adult acute myeloid leukemia. Ann Hematol 2006; 85: 108–112.

Lists of presentations from this thesis

Oral presentation

1. **Orathai Promsuwicha**, Wanna Thongnoppkhun, Somchai Wongkhuntee, Nittaya Uiprasert, Chintana Tocharoentanaphol, Chirayu Udomsakdi Auewarakul. Molecular Characterization of *PML/RAR*α Fusion Gene in Thai patients with Acute Promyelocytic leukemia (APL). Siriraj Medical Congress 2005 “Excellent Medical Practices for Better Quality of Life”, 4-8 July, 2005.

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Poster

1. **Orathai Promsuwicha**, Chintana Tocharoentanaphol, Somchai Wongkhantee, Chirayu Udomsakdi Auewarakul. IMMUNOLOGIC DIAGNOSIS OF ACUTE PROMYELOCYTIC LEUKEMIA (APL) WITH OR WITHOUT RECIPROCAL TRANSLOCATION OF CHROMOSOMES 15 AND 17. The 5th Princess Chulaphorn International Science Congress, 16-20 August 2004, Chulabhorn Research Institute (CRI).

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PUBLICATIONS:

1. **Orathai Promsuwicha**, Wanna Thongnoppakhun, Somchai Wongkhantee, Chintana Tocharoentanaphol, Chirayu U Auewarakul. Molecular Characterization of *PML/RAR*α Fusion Gene in Thai patients with Acute Promyelocytic Leukemia (APL) by nested RT-PCR. Thai Journal of Hematology and Transfusion Medicine, 2005; 15(4): 247-257.
2. Chirayu U. Auewarakul , Darat Lauhakirti, **Orathai Promsuwicha**, Chanya Munkhetvit. C-kit receptor tyrosine kinase (CD117) expression and its positive predictive value for the diagnosis of Thai adult acute myeloid leukemia. Ann Hematol 2006; 85: 108–112.

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