ANALYSIS OF *FLT3* MUTATIONS IN THAI PATIENTS WITH ACUTE MYELOID LEUKEMIA

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (IMMUNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2005

ISBN 974-04-6063-1

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Entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Master of Science (Immunology)
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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my major advisor, Assoc. Prof. Dr. Chirayu Auewarakul and my co-advisors, Assoc. Prof. Dr. Pa-thai Yenchitsommanus, Dr. Chanin Limwongse, and Prof. Dr. Napatawn Banchuin for their guidance, invaluable advice, instruction, supervision and other interactions that kept this work going throughout this study. I am equally grateful to the thesis committees and the external examiner, Dr. Chintana Tocharoentanaphol, for their excellent comments on my thesis.

My great thanks are given to the staffs at the Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, for the excellent care of the patients in this study and their help with leukemia sample collection and processing. Particularly, I am thankful to Ms. Orathai Promsuwicha, Ms. Kunjirat Promroeng and Ms. Darat Lauhakirte at the Hematologic Malignancies Laboratory, Division of Hematology, Department of Medicine for their hard work with flow cytometric analysis.

My sincere appreciation is expressed to Dr. Wanna Thongnoppakhun and all staffs in the Molecular Genetic Unit (MGU), Faculty of Medicine Siriraj Hospital for their kindness, continuous guidance, and encouragement throughout my study. I also appreciated the staffs and graduate students in the Department of Immunology, Medical Molecular Biology Unit (MMBU) and MGU for their friendship and kind support.

The financial supports from my family, Assoc. Professor Chirayu Auewarakul, Mahidol University Graduate Alumni Association, and Faculty of Medicine Siriraj Hospital Graduate Studies Scholarship are greatly appreciated.

Finally, I wish to thank all acute leukemia patients, without whom this study could not be possible. I sincerely hope that one day more of our Thai leukemia patients' lives could be saved, particularly with a less toxic and better treatment such as molecularly gene-targeting approach.

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ANALYSIS OF *FLT3* MUTATIONS IN THAI PATIENTS WITH ACUTE MYELOID LEUKEMIA

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ABSTRACT

Deregulation of receptor tyrosine kinases (RTKs) has been implicated in the molecular pathogenesis of leukemia. Mutation of FMS-like tyrosine kinase 3 (*FLT3*), a member of class III RTKs, has been reported to be the most frequent mutation in patients with acute myeloid leukemia (AML) in the West. No data currently exists regarding the incidence and characteristics of *FLT3* mutation in AML patients in Thailand or Southeast Asian countries. The objective of this study was to develop molecular methods to detect *FLT3* mutation whereby its incidence in adult AML patients in Thailand could subsequently be determined. The clinical and laboratory parameters of patients with wild-type *FLT3* and mutant *FLT3* were also analyzed.

Bone marrow or blood samples from 256 untreated adult AML patients were subject to RNA and DNA extraction. cDNA and genomic DNA were analyzed by RT-PCR and standard PCR, respectively, to detect *FLT3* internal tandem duplication (ITD) mutation. *FLT3*-tyrosine kinase domain (TKD) mutations in codon 835 and 836 were screened by RFLP analysis using *EcoR*V enzyme. Dual mutations were characterized by RT-PCR followed by RFLP. The aberrant products of *FLT3*-ITD and *FLT3*-D835 mutation were identified by sequencing.

Isolated ITD and TKD were identified in 63 (24.6%) and 8 (3.1%) of the cases, respectively. Dual mutations were found in 7 cases (2.7%). Sequence analysis of 14 ITD cases revealed that all duplicated fragments were in-frame duplications within the juxtamembrane region and the size varied from 7 to 67 amino acids. Interestingly, at least 1 SH2-binding motif including YFYV or YEYDLK was found. Six types of TKD mutations were identified with Asp835Tyr being the most frequent followed by Asp835His, Asp835Glu, Asp835Ala, and Ile836del. A *novel* mutation, Asp835del + Ile836Val was identified in one case. *FLT3* mutations were mostly found in patients with a normal karyotype. *FLT3*-ITD was significantly associated with high peripheral white blood cells counts and shorter overall survival than wild-type *FLT3*. Immunophenotypic profile did not differ between wild-type and mutant *FLT3*.

The molecular approach to detect and characterize *FLT3* mutations is now established in Thailand. *FLT3* mutation is a unique and frequent molecular target in Thai AML patients and its incidence is as high as that of the western countries. The prognostic impact of the *novel* mutation identified in this study remains to be determined in a larger population. The high prevalence of *FLT3* activating mutation in Thai patients makes it a potential target for novel therapy. It is hoped that the results of this study should be of value for future research and development of promising *FLT3* inhibitory drugs that will lead to cure of our patients who are suffering and dying from this tragic disease.

KEY WORDS : FMS-LIKE TYROSINE KINASE 3 (FLT3) / FLT3 MUTATIONS / ACUTE MYELOID LEUKEMIA (AML) / RECEPTOR TYROSINE KINASE (RTK)

113 P. ISBN 974-04-6063-1

การศึกษามิวเตชันของยีน *FLT3* ในผู้ป่วยไทยที่เป็นโรคมะเร็งเม็ดเลือดขาวมัยอีลอยด์ชนิดเฉียบพลัน
(ANALYSIS OF *FLT3* MUTATIONS IN THAI PATIENTS WITH ACUTE MYELOID LEUKEMIA)

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บทคัดย่อ

ความผิดปกติของ receptor tyrosine kinase (RTK) มีบทบาทสำคัญในการเกิดพยาธิสภาพของโรคมะเร็งเม็ดเลือดขาว ในต่างประเทศ มิวเตชันของยีน FMS-like tyrosine kinase 3 (*FLT3*) ซึ่งเป็นสมาชิกของ RTK กลุ่มที่ III พบได้บ่อยในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวมัยอีลอยด์ชนิดเฉียบพลัน แต่ยังไม่เคยมีผู้รายงานถึงความผิดปกติของยีนดังกล่าว ทั้งในประเทศไทยหรือในประเทศเพื่อนบ้านแถบเอเชียตะวันออกเฉียงใต้ ดังนั้นผู้ทดลองจึงได้ทำการทดลองและพัฒนาวิธีการตรวจหาความผิดปกติของยีน *FLT3* เพื่อตรวจหาอุบัติการณ์และชนิดของมิวเตชันนี้ในประเทศไทย รวมถึงศึกษาหาความสัมพันธ์ของยีนดังกล่าวกับอาการทางคลินิก และผลการตรวจทางห้องปฏิบัติการอื่นๆ ของผู้ป่วย

การศึกษาครั้งนี้ทำการศึกษาในผู้ป่วยคนไทยที่เป็นโรคมะเร็งเม็ดเลือดขาวมัยอีลอยด์ชนิดเฉียบพลันจำนวน 256 ราย โดยทำการสกัดสารพันธุกรรมชนิด DNA และ RNA จากสิ่งส่งตรวจที่เป็นเซลล์ไขกระดูกหรือเลือด cDNA และ genomic DNA ที่ได้จากผู้ป่วย จะถูกนำไปวิเคราะห์ด้วยวิธี reverse transcriptase-polymerase chain reaction (RT-PCR) และ PCR ตามลำดับ เพื่อตรวจหามิวเตชันของ *FLT3* ที่เป็น internal tandem duplication (*FLT3* ITD) ส่วนการตรวจหามิวเตชันของ tyrosine kinase domain (*FLT3*-TKD) ที่ตำแหน่ง codon 835 และ 836 จะใช้วิธีย่อยด้วยเอนไซม์ตัดจำเพาะ *EcoR*V การศึกษาวิเคราะห์ของ dual mutation จะใช้วิธี RT-PCR ตามด้วย restriction fragment length polymorphism (RFLP) และมีการใช้วิธีการโคลนนิ่งร่วมกับการประเมินลำดับเบส (sequencing) ในกรณีที่ตรวจพบมิวเตชันชนิดใหม่

จากการศึกษาพบมิวเตชันชนิด ITD จำนวน 63 ราย (ร้อยละ 24.7) และ TKD จำนวน 8 ราย (ร้อยละ 3.1) และตรวจพบมิวเตชันร่วมกันทั้ง 2 ชนิด ในผู้ป่วยคนเดียวกัน จำนวน 7 ราย (ร้อยละ 2.7) ผลจากการทดลองด้วยวิธีประเมินลำดับเบสในตัวอย่างที่เลือกแบบสุ่มที่เป็นมิวเตชันชนิด ITD พบว่าชิ้นส่วนของมิวเตชันมีการซ้ำซ้อนเป็นแบบ in-frame โดยเป็นชุดสาม แต่มีขนาดแตกต่างกันไปในผู้ป่วยแต่ละคน ตั้งแต่ 7 ถึง 67 กรดอะมิโน แต่ถึงอย่างไรก็ตามในทุกๆ ชิ้นส่วน จะตรวจพบชุดของลำดับกรดอะมิโนที่เป็นส่วนที่เรียกว่า SH2-binding motif คือพบ YFYV หรือ YEYDLK เสมอ สำหรับมิวเตชันชนิด TKD พบว่า Asp835Tyr เป็นมิวเตชันที่เกิดบ่อยที่สุด โดยตรวจพบจำนวน 7 ราย ในทั้งหมด 15 ราย รองลงมาคือ Asp835His, Asp835Glu, Asp835Ala, และ Ile836del นอกจากนี้ยังตรวจพบมิวเตชันชนิดใหม่ คือ Asp835del + Ile836Val ซึ่งไม่เคยมีผู้รายงานมาก่อนในวรรณกรรม การศึกษาความสัมพันธ์ของมิวเตชันกับข้อมูลทางคลินิกพบว่า ผู้ป่วยที่ตรวจพบมิวเตชัน มักจะตรวจไม่พบความผิดปกติทางโครโมโซมด้วยวิธีมาตรฐาน การตรวจพบมิวเตชันในตัวอย่างของผู้ป่วยยังมีความสัมพันธ์กับการเพิ่มจำนวนเซลล์เม็ดเลือดขาวในกระแสเลือดจำนวนมากอย่างมีนัยสำคัญ และยังสัมพันธ์กับการมีค่าอัตรารอดชีวิตที่ต่ำกว่าปกติด้วย สำหรับผลทางอิมมูโนฟีโนทัยป์พบว่าไม่มีความสัมพันธ์กับการมีมิวเตชัน

ขณะนี้ได้มีการพัฒนาและจัดตั้งวิธีการตรวจหาชนิดของมิวเตชันของยีน *FLT3* ขึ้นในประเทศไทย และพบว่ามิวเตชันชนิดนี้เป็นความผิดปกติทางอณูวิทยาที่มีความจำเพาะและพบได้บ่อยในคนไทยที่เป็นมะเร็งเม็ดเลือดขาวมัยอีลอยด์ชนิดเฉียบพลัน โดยมีอุบัติการณ์ใกล้เคียงกับประเทศทางตะวันตก ผู้ทดลองได้ค้นพบมิวเตชันชนิดใหม่ของยีน *FLT3* ซึ่งไม่เคยมีผู้รายงานมาก่อน ซึ่งควรจะต้องมีการศึกษาเพิ่มเติมในกลุ่มประชากรที่ใหญ่ขึ้น เพื่อดูความสำคัญของมิวเตชันใหม่นี้ในการบอกพยากรณ์โรคผู้ป่วยต่อไป การตรวจพบอุบัติการณ์ที่สูงของมิวเตชันของยีน *FLT3* ในคนไทยจะเป็นประโยชน์อย่างยิ่งในการพัฒนารักษาแบบใหม่ที่มุ่งเป้าแก้ไขความผิดปกติระดับโมเลกุลในเซลล์มะเร็งของผู้ป่วย โดยเฉพาะอย่างยิ่งการพัฒนายาที่มีความจำเพาะต่อยีน *FLT3* ซึ่งอาจนำไปสู่การรักษาที่หายขาดของผู้ป่วยมะเร็งเม็ดเลือดขาวมัยอีลอยด์ชนิดเฉียบพลันที่กำลังทนทุกข์ทรมานและเสียชีวิตจากโรคร้ายแรงนี้

113 หน้า . ISBN 974-04-6063-1

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LIST OF ABBREVIATIONS

Abbreviations		Term
bp	=	base pair
°C	=	degree Celsius
dATP	=	deoxyadenosine-5’-triphosphate
dCTP	=	deoxycytosine-5’-triphosphate
dGTP	=	deoxyguanosine-5’-triphosphate
dTTP	=	deoxythymidine-5’-triphosphate
EDTA	=	ethylenediamine tetraacetic acid
ITD	=	internal tandem duplication
kb	=	kilobase
M	=	molar
mg	=	milligram
ml	=	millilitre
mM	=	millimolar
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
ng	=	nanogram
nm	=	nanomolar
nt	=	nucleotide
OD	=	optical density
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
pmol	=	picomolar
RT-PCR	=	reverse transcriptase polymerase chain reaction

LIST OF ABBREVIATIONS (cont.)

Abbreviations		Term
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

POSTER PRESENTATIONS

1. Chirayu U Auewarakul, Narongrit Sritana, Wanna Thongnoppakhun, Pa-thai Yenchitsomanas, Chanin Limwongse. Rapid molecular detection of *FLT3* mutations in Thai patients with acute myeloid leukemia (AML). The 10th ASEAN conference in Medical Laboratory Technology, 26-30 April 2004, ASEAN Association of Medical Laboratory Technologist (AAMLT).
2. Chirayu U Auewarakul, Narongrit Sritana, Wanna Thongnoppakhun, Chanin Limwongse, Pa-thai Yenchitsomanas. Identification of *FLT3* mutations in Thai patients with acute myeloid leukemia (AML). The 5th Princess Chulabhorn International Science Congress, 16-20 August 2004, Chulabhorn Research Institute (CRI).

CHAPTER I

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells in the marrow and an arrest on their maturation. These immature myeloid cells, also called blasts, accumulate and rapidly replace bone marrow, resulting in decreased production of red cells, white cells, and platelets (1). Loss of normal bone marrow functions results in multiple complications including anemia, infection, and bleeding. Occasionally, blasts may invade the lymphatic system, spleen, and other vital organs. Left untreated, AML inevitably will lead to death. In general, acute leukemia can be diagnosed by morphology, cytochemistry, cytogenetics, and cell surface marker analysis. The most popular classification of acute leukemia was the French-American-British (FAB) Classification that was developed in 1976 and was based solely on the morphological and histochemical studies of the leukemic blasts. The FAB Classification, however, has limitations since it cannot precisely predict the leukemic cell lineages and the clinical outcome of the patients. A more recent classification has been proposed in 2001 by the World Health Organization (WHO), which takes into account the importance of karyotypic and genetic abnormalities of leukemic cells. Based on karyotype analysis, 3 different risk groups of AML patients can be distinguished (2, 3) as shown in Table 1.

Table 1 Risk stratification of AML patients according to leukemia karyotype

Karyotype groups	**Cytogenetic abnormalities**
Good risk	t(15;17), t(8;21), inv(16) or t(16;16)
Standard risk	Normal and all other cytogenetic abnormalities
Poor risk	t(9;22), -5, -7, complex karyotype, 11q23 abnormalities

The good risk group consists of the patients with either t(15;17), t(8;21), inv(16) or t(16;16) whereas the poor risk group includes patients who have unfavorable chromosomes such as chromosome 5/7 abnormalities, 11q23 abnormalities or complex karyotypes. The standard risk group is a heterogenous group of patients who lacked any specific karyotypic markers. Current research focuses on the identification of molecular genomic and proteomic markers that may be useful for further subclassification of this latter group of patients.

The growth and differentiation of hematopoietic cells are governed by the concerted action of growth factors and their receptors. One of these receptors, Fms-like tyrosine kinase 3 (FLT3) (4), also called stem cell kinase 1 (STK1) or fetal liver kinase 2 (flk2) (5) belongs to a group of class 3 receptor tyrosine kinases (RTKs) which also consists of c-Kit, PDGF-R, and c-fms (6). This class of RTK is characterized by five immunoglobulin-like domains in the extracellular ligand-binding region, transmembrane and juxtamembrane (JM) domain and two TK domains interrupted by kinase insert and C-terminal tail. The *FLT3* gene is located at chromosome 13q12 and consists of 24 exons (previously reported as 21) (4). RTKs are normally activated by receptor-specific ligands, which bind to the extracellular domains and promote phosphorylation of the intracellular tyrosine kinase domains (7). FLT3 is predominantly expressed on haematopoietic progenitor cells in the bone marrow, thymus and lymph nodes (8), but is also found on other tissues such as placenta, brain, cerebellum and gonads (8, 9). Interestingly, FLT3 is also expressed at high levels in 70% to 100% of cases of AML and in a high percentage of acute lymphoid leukemia (ALL) cases. These data indicate that FLT3 expression may play a role in the survival or proliferation of leukemic blasts.

Two distinct types of *FLT3* mutations have now been identified in the subsets of AML cases that express FLT3; internal tandem duplication (ITD) mutations within the juxtamembrane region and point mutations at aspartate residue 835 (D835 or Asp835) within the kinase domain. Both types of mutation constitutively activate the tyrosine kinase activity of FLT3 in experimental systems. *FLT3*-ITD mutations cluster in exon 14 and exon 15 (previously 11 and 12) of human *FLT3* gene, a part that code for juxtamembrane domain of FLT3 protein (10). This alteration induces constitutive activation of the protein and leads to activation of downstream signaling molecules,

including STATs, Ras, and MAP kinase when transfected into 32D or BA/F3 cells (11). Many studies have reported that the presence of the *FLT3*-ITDs is associated with statistically significant worse clinical outcomes compared with patients not harboring this mutation (12, 13). Because the mutation is not always a single duplication but often contains foreign sequence or addition of extra nucleotides, some researchers will not use the term ITDs but will refer to it as *FLT3*-length mutation (*FLT3*-LM) (2). More recently, point mutations in codon 835 of *FLT3* gene have been described in approximately 7% of patient with AML (14). These mutations induce conformational changes of A-loop, which results in opening of the catalytic pocket and constitutive active kinase activity. The incidence of *FLT3*-mutations in AML appears to be up to 41% (17-34% *FLT3*-ITD mutation, 7% Asp835 mutation). Very recently, a series of novel mutations within the activation loop were identified in AML patients including deletion of isoleucine 836 (I836del) and substitution of isoleucine 836 to methionine with addition of arginine inserted after codon 836 (I836M+R) (15), and a 6 bp insertion in the activation loop between codons 840 and 841 of *FLT3* (FLT3-840GS) (16). The impact of these mutations on FLT3 receptor signaling activity has not yet been investigated.

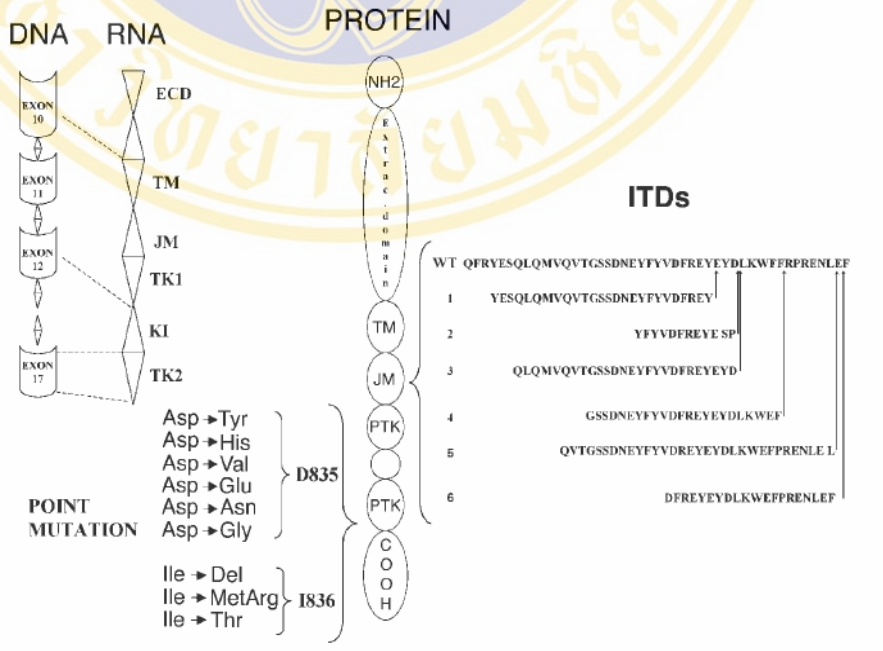

Figure 1 Schematic representation of the FLT3 receptor kinase domains with point mutations and some types of ITD. (From Martinelli G et al. 2002)

Current therapy using cytologic agents such as cytosine arabinosine (AraC) and daunorubicin cures approximately 30 % of patients with AML. Most published studies demonstrate that the *FLT3*-activating mutations were associated with a distinctly worse prognosis. In one study, the cure rate with a *FLT3* mutation was reported to be as low as 7% compared to a cure rate of 44% without mutation (17). Recent clinical success with the BCR-ABL inhibitor STI-571 (imanitib mesylate) in chronic myeloid leukemia (CML), suggest that therapies targeted to specific activated kinase may be an effective approach in certain malignancies. The high frequency of *FLT3* alterations in AML has fostered investigations into molecularly targeted treatments and the development of agents directed against this receptor, such as hervimycin A or CEP-701 (18). These new drugs could selectively act against leukemic cells with activated FLT3. Very recently, kinase inhibitors were found to have distinct inhibitory potencies against different activating FLT3 receptor mutants (19). These results suggest that it may be useful to determine the exact kind of *FLT3* mutations when applying receptor kinase inhibitors in clinical trials.

At present, no data exists in Thailand and other Southeast Asian countries with respect to the incidence, type, and prognostic impact of *FLT3* mutations despite the fact that AML is one of the most common hematologic malignancies associated with the greatest mortality and morbidity in adult populations in these countries. Strikingly, Thai patients appeared to develop acute leukemia at a much younger age than those in the west, with the median age at onset of only 35-45. Genotypic analysis of *FLT3* gene may help to elucidate the molecular pathogenesis of AML that arises in Thai population. In this thesis, I set out to explore the frequency and characteristics of *FLT3* mutations in our unique ethnic population. It is hoped that the results would help us not only to understand the molecular pathogenesis of AML but also guide us how to manage our patients with this tragic disease at the time when the molecularly targeted drugs such as FLT3 tyrosine kinase inhibitors become available in Thailand.

CHAPTER II

OBJECTIVES

1. To determine the frequency and types of *FLT3* mutations (*FLT3*-ITD and *FLT3*-TKD mutations) in Thai AML patients.
2. To evaluate the association of *FLT3* mutation with karyotype, immunophenotype and clinical outcome of these patients.

CHAPTER III
LITERATURE REVIEW

1. Leukemia

1.1 Definition and cause of leukemia

Leukemia is a cancer of blood cells. When leukemia develops, the body produces large numbers of abnormal blood cells. The cause of most leukemia is unknown. There are several factors associated with increased leukemia risk, including hereditary and congenital genetic disorders and environmental factors including exposure to radiation, chemicals or drugs and viral infections.

1.1.1 Hereditary genetic disorders

Several hereditary and congenital disorders are associated with increased leukemia risk, including Fanconi anemia, Bloom syndrome, ataxia telangiectasia, Down's syndrome and other congenital syndromes.

Fanconi anemia is an autosomal recessive disorder characterized by progressive bone marrow failure. The molecular defect is thought to be an inability to repair DNA crossing(20). Acute myeloid leukemia (AML) is common in patients with Fanconi anemia surviving bone marrow failure. In recent studies actuarial incidence exceeds 50% (21). Some patients develop AML directly or after antecedent myelodysplasia, without a clinically apparent aplastic phase.

Bloom syndrome is another autosomal recessive disorder associated with increased leukemia risk (22). The molecular basis is thought to be deficient DNA ligase (23). In contrast to Fanconi anemia, children with Bloom syndrome develop acute lymphoblastic leukemia (ALL) as well as AML.

Ataxia telangiectasia is an autosomal recessive disorder characterized by cerebral and vascular abnormalities (24). The molecular basis involves a subtle abnormality in DNA repair (25). Other abnormalities are also reported, such as impaired cysteine transport. Leukemia in ataxia telangiectasia are exclusively

lymphoid, mostly T cells. Clones of T-cells with abnormalities of one of both chromosomes 14, typically involving the beta T-cell receptor locus, are common in patients with ataxia telangiectasia.

Children with Down's syndrome also have an increased risk of developing ALL and AML (26). The actuarial risk is reported to be about 1.5%. Most cases are acute megakaryocytic leukemia (AML-M7). Leukemia developing in Down's children is clonal and in Down's mosaic they develops only in trisomic cells. Trisomy 21 is also a common acquired genetic abnormality in AML without Down's syndrome.

Recently, acquired mutations of the hematopoietic transcription factor, GATA binding protein-1 (*GATA1*) were found in megakaryoblasts from patients with acute megakaryoblastic leukemia (AMKL) and Down's syndrome, but not in cases of AMKL arising in individuals lacking trisomy 21. These *GATA1* mutations were restricted to the leukemic clone and were not found in normal remission samples. The *GATA1* mutations were also reported in nearly all individuals with Down syndrome with transient myeloproliferative disorder (TMD, also called transient leukemia). It was proposed that *GATA1* mutations may have occurred in the hematopoietic stem cells (HSCs) independently of the trisomy 21 defect, and imparted a clonal advantage which, in cooperation with the increased dosage of chromosome 21 genes, contributed to leukemogenesis.

1.1.2 Radiation, chemicals and drugs

Exposure to radiation, chemicals and drugs are possible causes of leukemia. The best examples of radiation-related leukemogenesis were demonstrated in patients who were previously exposed to atomic bomb radiation or received radiation therapy for malignant or non-malignant diseases and children who were exposed to diagnostic X-ray in utero.

Several forms of leukemia were increased after atomic bomb exposure, inclucing AML, ALL and chronic myelogenous leukemia (CML) (27, 28). There was no correlation between age at exposure and the type of leukemia. Several reports of increased risk of leukemias, mostly AML and rarely CML, in patients treated with radiation therapy for breast, cervical, or endometrial cancers and lymphoma. Children

exposed to diagnostic X-ray in utero usually develop leukemia, particularly ALL (29), within 2 years after exposure.

Benzene and several chemicals were also indirectly linked to leukemia by epidemiologic studies. Many chemotherapeutic drugs can also cause DNA damage and induce leukemia. Therapy-related leukemias are mostly AML.

1.1.3 Virus-related leukemias

Viruses are a common cause of leukemia in animals, including primates. However, the search for a viral etiology of most human leukemia has been unsuccessful. Although considerable epidemiologic data suggest that ALL in children reflects an uncommon response to a common infection (30, 31), no specific virus has been detected. There are, however, some well documented virus-related types of lymphoid malignancies, adult T-cell leukemia and human T-cell lymphotropic virus 1 (HTLV-1) and Burkitt lymphoma-leukemia and Epstein Bar virus (EBV).

1.2 Types of leukemia

There are several types of leukemia. They are group in two ways. One way is by how it develops and gets worse. The other way is by the type of blood cells that is affected.

Leukemia is either acute or chronic. In acute leukemia, the abnormal blood cells are blasts that remain very immature and cannot carry out their normal functions. The number of blasts increases rapidly, and the patients get worse quickly. In chronic leukemia, some blast cells are present, but in general, these cells are more mature and can carry out some of their normal functions. Also, the number of blasts in chronic leukemia increases less rapidly than in acute leukemia. As a result, chronic leukemia gets worse gradually.

Leukemia can arise in either of the two main types of white blood cells, lymphoid cells or myeloid cells. When leukemia affects lymphoid cells, it is called lymphoid or lymphocytic leukemia. When myeloid cells are affected, the disease is called myeloid or myelogenous leukemia. The most common types of leukemia are shown below.

i. Acute lymphoid leukemia (ALL) is the most common type of leukemia in young children. This disease also affects adults, especially those aged 65 and older.
ii. Acute myeloid leukemia (AML) occurs predominantly in adults and rarely in children.
iii. Chronic lymphocytic leukemia (CLL) affects older adults, especially those who are over the age of 60. It sometimes occurs in younger adults, but never affects young children.
iv. Chronic myeloid leukemia (CML) occurs mainly in middle-aged and older adults. A very small number of children also develop this disease

Moreover, there are also other uncommon types of leukemia such as hairy cell leukemia, plasma cell leukemia and prolymphocytic leukemia.

1.3 Acute myeloid leukemia (AML)

1.3.1 Epidemiology of AML

Approximately 10,500 new cases of AML are diagnosed annually in the United States, accounting for less than 1% of all cancer and 34% of all leukemias (32). Age-specific incidences differ dramatically between ALL and AML, with a median patient age at diagnosis of 10 years and 65 years, respectively. The incidence of AML is rare below the age of 40 but increases progressively with age, from approximately 1 per 100,000 at age 40 to more than 15 per 100,000 at age 75 and older (32). AML has a slight male predominance (1.2:1.0) in the western countries.

1.3.2 Clinical presentation of AML

The clinical signs and symptoms of AML are diverse and nonspecific, but they are usually directly attributable to the leukemic infiltration of the bone marrow, with resultant cytopenia. Typically, patients present with signs and symptoms of fatigue, hemorrhage, infections or fever due to decreases in red cells, platelets, or white cells, respectively. Pallor, fatigue, and dyspnea on exertion are common. Leukemic infiltration of various tissues, including the liver (hepatomegaly), spleen (splenomegaly), skin (leukemia cutis), lymp nodes (lymphadenopathy), bone (bone

pain), gingival, and central nervous system, can also occur. An isolated mass of leukemic blasts is usually referred to as a granulocytic sarcoma. Hyperleukocytosis (more than 100,000 white cells per cubic millimeter) can lead to symptoms of leukostasis, such as pulmonary and cerebrovascular dysfunction or bleeding.

1.3.3 Diagnosis of AML

The primary diagnosis of AML rests on the morphologic identification of leukemic myeloblasts in preparations of peripheral blood and bone marrow stained with Wright-Giemsa. The myeloblasts have round to irregular nuclei, distinct nucleoli, and varying amount of cytoplasm. The cytoplasm frequently contains fine azurophilic granules and a variable number of Auer bodies of rods. The presence of more than 20% leukemic blasts in a bone marrow aspirate is required for a definitive diagnosis of acute leukemia. Before therapy is initiated, however, several critical diagnostic distinctions must be made. AML must be distinguished from ALL, myelodysplastic syndrome (MDS), or AML arising in the setting of MDS, because therapeutic strategies and prognosis vary considerably for these diseases. AML can be distinguished from ALL by demonstration of definitive commitment to myeloid lineage through judicious use of morphologic, immunohistochemical, and immunologic methods (33-35).

Once a diagnosis of AML is made, the morphologic and genetic subtype must be identified. Immunologic methods have recently been incorporated into the diagnostic criteria. The most popular classification of AML in the 1970's was the French-American-British (FAB) Classification that relies mainly on the morphological and histochemical analysis of leukemic cells. Recently, a new classification developed by the World Health Orgnaization (WHO) has emphazises on the diagnosis of AML based on cytogenetic and molecular abnormalities. Cytogenetic analysis of leukemic blasts has resulted in the identification of many nonrandom clonal chromosomal aberrations in a large percentage of patients with AML (36, 37). However, not all FAB subgroups have specific cytogenetic abnormalities. The combination of morphologic, immunologic, and genetically based diagnostic approaches not only makes it possible to accurately diagnose acute leukemia but also to guide the therapy according to the specific subtype. Moreover, the immunologic

and molecular abnormalities also provide unique markers with which to monitor a patient's response to therapy.

1.3.4 Chromosome abnormalities in AML

In the majority of patients with AML, acquired clonal chromosome aberrations can be observed. Numerous recurrent chromosome abnormalities have been discovered in AML (Table 2). These findings at the chromosomal level have paved the way for molecular identification of genes involved in the process of leukemogenesis. The identification of specific chromosomal abnormalities and their correlation with cytomorphologic features, immunophenotype, and clinical outcome have led to a new understanding of AML as a heterogeneous group of distinct biologic entities. The importance of chromosome abnormalities in AML for classification and for the understanding of pathogenetic mechanisms is increasingly appreciated in the clinical context, and the new WHO Classification of AML uses cytogenetic abnormalities as a major criterion.

Table 2 Examples of chromosome abnormalities in acute myeloid leukemia (AML)

Abnormalities	Fusion genes	FAB subtype	Frequency	
			Children (%)	Adults (%)
t(8;21)(q22;q22)	AML1-ETO	M1/M2	10-15	8-12
inv(16)(p13q22)	CBFβ-MYH11	M4eo	6-12	8-12
t(15;17)(q22;q21)	PML-RARα	M3/M3v	8-15	8-10
t(9;11)(p22;q23)	MLL-AF9	M5a	8-10	1-2
t(3;21)(q26;q22)	AML1-EAPEVI1	-	1	<1
t(6;9)(p23;q34)	DEK-CAN	M1/M2	1-2	rare
inv(3)(q21q26)	EVI?-?	-	<1	1-2
t(1;22)(p13;q13)		M7	2	-
+8		-	1-4	3-5
+11		M1/M2	-	<1
Complex		-	6	10-20

* Data from Schoch *et al*, 2002 (38)

Primary chromosome aberrations are frequently found as the sole karyotypic abnormality and are often specifically associated with a particular AML subtype. On average, 55% of AML patients with karyotype abnormalities have only one rearrangement (15% to 20% have gain or loss of a single chromosome). These primary abnormalities are thought to play an essential role in the early stages of leukemogenesis. On the contrary, secondary chromosome abnormalities appear to play an important role in the progression of the disease, but they are rarely or never found alone. Although less specific than the primary changes, secondary aberrations nevertheless demonstrate nonrandom features with distribution patterns that appear to be dependent on the primary abnormality. The primary aberrations are often balanced re-arrangements such as translocations or inversions while secondary aberrations usually involve genomic imbalances (gains or losses of whole chromosomes, deletions, or unbalanced translocations).

1.3.4.1 Primary chromosome aberrations

Two major types of primary chromosome aberrations can be distinguished: 1) balanced structural abnormalities (reciprocal translocations, inversions, and insertions) which lead to a leukemia-specific fusion gene; and 2) unbalanced aberrations (trisomies, monosomies, deletions, unbalanced translocations, and isochromosomes) which lead to gain or loss of chromosomal materials. The most frequent balanced chromosome abnormalities in AML and genes affected by their formation are t(8;21)(q22;q22)AML1/ETO, t(15;17)(q22;q12)PML-RARα, inv(16)(p13q22)/ t(16;16)(p13;q22)CBFβ-MYH11, and 11q23 abnormalities-/MLL-rearrangements. 15% to 25% of all AML cases were reported to carry these abnormalities. Less common ones are inv(3)(q21q26)/t(3;3)(q21;q26), t(6;9) (p23;q34)DEK-CAN, t(8;16)(p11;p13) MOZ-CBP, t(1;3)(p36;q21), t(1;22) (p13;q13), t(3;21)(q26;q22), t(7;11) (p15;p15), t(11;17)(q23;q25), and t(16;21) (p11;q22). Several unbalanced abnormalities, including gains and losses of whole chromosomes or deletions, are also thought to be primary abnormalities, but the pathogenetic mechanism is presently not resolved. The most common unbalanced abnormalities are deletion 5q, monosomy 7, deletion 7q, trisomy 8, deletion 9q, trisomy 11, trisomy 13, and trisomy 21. 10-20% of AML patients have complex aberrant karyotypes, which

are associated with a very poor prognosis. The definition of 'complex aberrant karyotype' varies depending on the study groups. It is usually defined as consisting of at least three cytogenetic abnormalities. The real complex aberrant karyotype shows unbalanced abnormalities with loss of material of 5q, 7q, and/or 17p (38).

1.3.4.2 Secondary chromosome aberrations

Approximately 45% of AML patients with an aberrant karyotype have two or more karyotype abnormalities. The secondary chromosome aberrations develop in cells already carrying a primary abnormality. In 70% to 80% of patients with t(8;21)(q22;q22), additional aberrations are observed. The most common secondary aberration is the loss of a sex chromosome. Other aberrations observed are a trisomy 8 and a deletion of the long arm of chromosome 9. Thirty percent to 50% of patients with inv(16) show additional chromosome aberrations. Most often observed are trisomies 22, 21, or 8. The frequency of secondary chromosome aberrations in patients with t(15;17) is 30 to 40%, with trisomy 8 and an isochromosome of the derivative chromosome 17 observed most often. Conflicting data on the prognostic impact of secondary aberrations have been published. Although no influence on prognosis of secondary abnormalities in patients with t(8;21)(q22), inv(16)(p13q22), or t(15;17)(q22;q12) were reported in the large Medical Research Council (MRC) 10 trial and some smaller studies, a negative prognostic impact of additional abnormalities was reported for patients with t(8;21) and t(15;17) in one study each. Further study is needed to assess the clinical consequences of secondary aberrations in AML.

The presence of an exclusively normal karyotype in a diagnostic marrow sample does not mean that cytogenetically normal leukemic blasts harbor no acquired genetic alterations. A variety of genetic abnormalities discernible only by molecular genetic techniques, such as Southern analysis, RT-PCR or direct sequencing, have been reported. These include partial tandem duplication of the *MLL* gene (PTD MLL) internal tandem duplication of the *FLT3* gene (*FLT3*-ITD) activating point mutations of D835 within the activation loop of the FLT3 gene (FLT3-TKD mutation) point mutations of the *NRAS, CEBPA,* and *RUNX1 (AML1)* genes deletions and point mutations of the PU.1 gene, homozygous deletions resulting in null

genotypes at the glutathione S-transferase, GSTM1 and GSTT1 loci and overexpression of the BAALC, and EVI1 genes (38).

1.3.5 Genetics and prognostic factors of AML

The importance of karyotype in defining the natural history and response to therapy in acute leukemia has recently been recognized (39, 40). Perhaps the most supportive evidence in this regard is the increased prevalence of various chromosomal abnormalities in older adults compared with younger patients with AML. The genetic mutations most often associated with treatment failure in young patients with AML (eg. abnormalities of chromosome 5 or 7 complex karyotypes) are considerable more common in the elderly, occurring in 32% to 57% of patients. Conversely, all of the "favorable" cytogenetic abnormalities, such as t(8;21), t(15;17) or inv(16), are more common in younger subjects and are responsible in a part for their better disease-free survival (40).

Results from the UK Medical Research Council AML 11 trial, in which the median age of patients was 66 years, have identified the following prognostic groups (41).

i. Favorable prognosis group [t(15;17), t(8;21), and inv(16) or t(16;16)] has complete remission (CR) rate of 72% and 5-years overall survival (OS) rate of 34%.
ii. Intermediate prognosis group (normal karyotype or noncomplex karyotype) has CR rate of 53% to 63% and 5-year OS rate of 10% to 15%
iii. Poor prognosis group (complex karyotype, -5/-7 abnormalities) has CR rate of 26% and 5-year OS rate of 2%

These categories are similar to those that have been defined for younger adults, although the survival rates are inferior. A report from the Southwest Oncology Group (SWOG) included 211 elderly patients with AML (mean age = 68 years) (42). Unfavorable cytogenetics were present in 32%, these patients had a much lower CR rate than those with favorable of intermediate cytogenetics (21% vs 55%). Similar results have been reported from the German AML Cooperative Group (43, 44) and from a large Swedish study (45).

1.3.6 Multistep pathogenesis of AML

Much information has been gained through the cloning and characterization of genes from the chromosomal translocation breakpoints as described above (46). There is convincing evidence that gene rearrangements in AML involving transcription factors are necessary for the onset of the disease, but there is also equally compelling evidence that these events are not sufficient to cause a full leukemic phenotype, as evidenced in part by the long latencies required for disease development in the murine models of the disease. These data support the hypothesis that multiple genetic abnormalities rather than a unique molecular defect are necessary for a phenotype of AML (47). An emerging paradigm is the co-operation between transcription factor fusions and other molecular events such mutations in the FLT3 and c-kit receptor tyrosine kinases (48), ras and NF-1 mutations (49), aberrant activation of the Jak/Stat pathway, epigenetic changes in the promoter regions of tumor suppressor genes and dysregulation of the cytokine network genes in the pathogenesis of AML (50). In such a model, additional abnormalities confer proliferation and/or anti-apoptotic activity to the hematopoietic cells, while the transcription fusion impairs normal differentiation pathways.

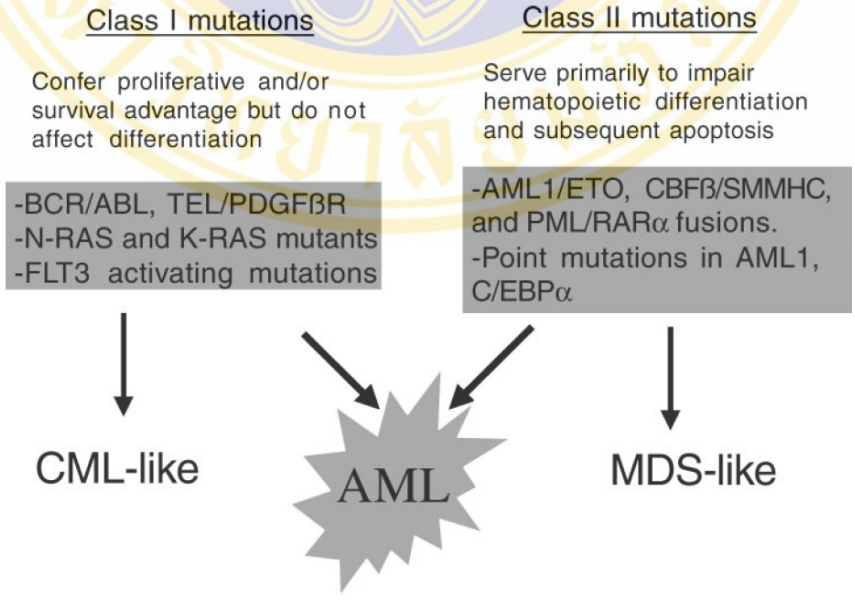

Figure 2 Two classes of cooperating mutations hypothesized to cause acute myeloid leukemia (AML) (Gilliland et al, 2002)

1.3.7 Animal model in AML

Several different murine models of leukemia provide convincing evidence for a requirement for a second mutation for fusion genes cloned from AML patients. An elegant genetic strategy using a conditional allele has been utilized to achieve expression of the AML1/ETO fusion protein in adult hematopoietic progenitors. However, AML does not develop in these animals unless chemical mutagens, such as ethyl-nitrosourea (ENU), are used. Similarly, low levels of expression of CBFß/SMMHC can be achieved in hematopoietic progenitors in chimeric animals. However, these animals only develop AML after long latency unless ENU is administered. PML/RARa expression in transgenic mice under the control of the Cathepsin G promoter results in AML with some features of acute promyelocytic leukemia in humans. In this model, there is long latency of disease, incomplete penetrance, and acquisition of karyotypic abnormalities with progression to AML, all indicative of a requirement for second mutations. Furthermore, activating mutations in receptor tyrosine kinases, for example, TEL/PDGFbR and FLT3 present in approximately 30% of AML cases, were found to cooperate with AML1/ETO, NUP98/HOXA9 t(7;11) and PML/RARa t(15;17) causing AML-M2 type leukaemia, AML and APL, respectively, in transduced mice. Concurrently, cotransduction of murine HSCs with mutated tyrosine kinase BCR/ABL and translocation of NUP98/HOXA9 resulted in AML following transplantation into syngenic mice. The technique of retroviral transduction and transplantation was also applied to the genesis of MLL/ELL t(11;19) and to AML1/MDS1/EVI1 t(3;21) murine models. Induced disease states which recapitulated that of the equivalent human phenotype but with long disease latencies were observed, suggesting that translocated genes, although crucial for AML induction, obligate acquisition of further genetic abnormalities to induce advanced disease. Subsequently, cotransduction and transplantation of doubly BCR/ABL-AML1/MDS1/EVI1, Hoxa9-Meis1 and Hoxa9-E2A/Pbx1 transfected cells into syngeneic recipients yielded aggressive AML diseases of short latencies, in comparison to singly transducted and transplanted animals. Finally, a major part of the studies with transgenic mice, knock-in mice and retroviral transduction conclude that leukemogenesis in AML is a multi-step process (47).

2. Receptor tyrosine kinases (RTKs)

1.1 The discovery of RTKs and RTKs structures

More than 20 years ago, the first primary structure of receptor tyrosine kinase, the epidermal growth factor receptor (EGFR), was elucidated. This event led to many laboratories interested to study the molecular mechanisms of RTK function. Moreover, the substantial studies have been made in understanding the role of RTK in the signaling pathway that involved in cellular process, such as migration, proliferation, metabolism, differentiation and survival, as well as, the intercellular communication during cell development. In normal cells, the RTK activity is tightly controlled. However, RTK become oncoproteins when they are mutated or structurally altered.

The beginning of the RTK discoveries start from the year 1952, when Rita Levi Moutalcini discovered a secreted factor in mouse tumor cells that potently promoted neurite outgrowth in chicken embryos (51). This protein called nerve growth factor (NGF) was purified from snake venom and mouse salivary gland extracts (52, 53). Five year later, Cohen isolated and characterized the bioresponse-mediated substance that was called epidermal growth factor (EGF) (54), as it stimulated the proliferation of epithelial cells (55).

In 1978, Cohen and coworker identified EGFR as 170-kDa membrane components that showed increased ^{32}P incorporation in response to EGF treatment in A-431 epidermoid carcinoma cells (56). It was proposed at that time the phosphorylation of membrane components and membrane association proteins might be crucial events in the generation of intracellular signals that regulate proliferation. The concept of signal generation by tyrosine phosphorylation gained further experimental support in the early 1980, when three reports showed that EGFR (57) receptor, the insulin receptor (INSR) (58), and the platelet-derived growth factor receptor (PDGFR) (59) are protein tyrosine kinases that can be activated by their respective ligands.

The development of molecular cloning in the mid 1970s allowed the identification of the cDNAs that encode important physiological peptide hormones and growth factors, such as insulin, EGF, insulin-like growth factor 2 (IGF2), nerve growth factor (NGF), platelet-derived growth factor (PDGF) and transforming growth factor-alpha (TGF-alpha) (60). This led to their sequencing and the determination of

their amino acid sequences and subsequently, to manufacture medically important hormones in late 1970s. Until 1984, a team of collaborators from Imperial Cancer Research Fund (ICRF) isolated and characterized the cDNA sequence of human EGFR - the prototypical RTK - from normal placenta cells and A431 tumor cells (61). The characterization of EGFR cDNA provided the first complete amino acid sequence of a cell surface receptor that had signal generating ability and provided detailed insights into its molecular architecture.

Human EGFR was found to be a large glycoprotein with a modular structure. It contains an extracellular ligand-binding domain connected to the cytoplasmic domain by a transmembrane helix. The cytoplasmic part consists of a juxtamembrane region, a tyrosine kinase region that is flanked by non-catalytic regulatory regions. The EGFR cDNA cloning project (61) provided two important discoveries. First, Southern blot analysis with an EGFR cDNA probe showed increasing amplification of EGFR (25 folds) in human A431 epidermal carcinoma cells. This is a prototypical genetic abnormality that should prove to be highly relevant to future developments. Second, the screening of cDNA libraries yielded sequences that were related to EGFR but clearly distinct, which gained importance in subsequent studies (60).

In the years following the clarification of the primary structure of EGFR, the nucleotide sequences and amino acid sequences of several other RTK were reported. These included INSR, the IGF1 receptor (IGF1R) and PDGFR, as well as those that are encoded by proto-oncogenes, KTT and FMS. This series of studies confirmed that RTKs are highly related in structure and share a domain arrangement that is very similar to that of EGFR

1.2 Classification of RTKs

The human genome is believed to contain 518 putative protein kinase genes, of which 58 are of the tyrosine receptor type (62). The latter, transmembrane enzymes involved in ligand binding and signal transduction at the cell surface, have been grouped into 20 classes, or subfamilies, based on their kinase domain sequence, a feature that also parallels their overall structure (63) (Figure 3).

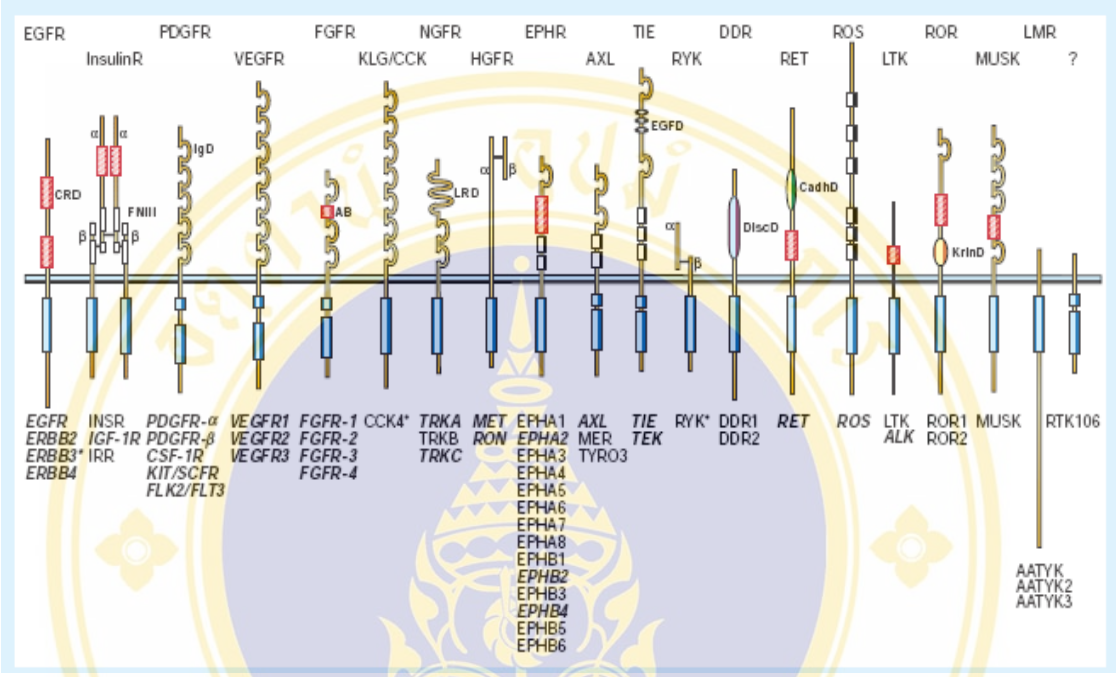

Figure 3 Human receptor protein-tyrosine kinases. The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. Abbreviations of the prototypic receptors: EGFR, epidermal growth factor receptor; InsR, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR; vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; KLG/CCK, colon carcinoma kinase; NGFR, nerve growth factor receptor; HGFR, hepatocyte growth factor receptor, EphR, ephrin receptor; Axl, a Tyro3 PTK; TIE, tyrosine kinase receptor in endothelial cells; RYK, receptor related to tyrosine kinases; DDR, discoidin domain receptor; Ret, rearranged during transfection; ROS, RPTK expressed in some epithelial cell types; LTK, leukocyte tyrosine kinase; ROR, receptor orphan; MuSK, muscle-specific kinase; LMR, Lemur. Other abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols a and b denote distinct RPTK subunits. RPTK members in bold and italic type are implicated in human malignancies. An asterisk indicates that the member is devoid of intrinsic kinase activity. (Blume-Jensen P *et al*, 2001) (64)

2.3 RTKs in normal and malignant haematopoiesis

It is now known that proteins from a number of different RTK classes (for example, class II – V, VII, XI and XIV) are expressed on normal haematopoietic tissue, and that most have been linked to the pathogenesis of leukemia.

2.3.1 RTK class II (Insulin receptor family)

The class II RTK exists as heterotetramer that is different from the majority of RTK that are monomers. Two polypeptide chains are entirely extracellular with a disulfide bridge linked to each other and to two identical subunits with membrane spanning and tyrosine kinase domains (Figure 3). The insulin-like growth factors regulate the proliferation and differentiation of haematopoietic cells via specific receptors located on erythrocytes, monocytes, as well as B and T lymphocyte. Hypersensitivity to IGF-1 has been implicated as an underlying cause of polycythemia vera (65) while loss of imprinting of IGF-11 is found in AML and myelodysplastic syndrome (66).

2.3.2 RTK class III (PDGF receptor family)

Class III RTKs are characterized by five immunoglobulin-like domains in the extracellular ligand-binding region and include c-KIT, FLT3, PDGFR and c-FMS (67). This group of RTK plays a crucial role in normal haematopoiesis with the probable exception of PDGFRα. The details of this class are discussed in the next part.

2.3.3 RTK class IV (FGF receptor family)

The FGF receptor family is characterized by three immunoglobulin-like domains in the extracellular part (Figure 3). This family comprises four gene, FGFR-1, FGFR-2, FGFR-3, and FGFR-4. The FGFR-1 gene is the hallmark of stem cell myeloproliferative disorder associated with chromosomal rearrangments involving 8p11 (68, 69). Somatic mutations resulting in FGFR3 activation have been reported in a small number of multiple myeloma cases that express the t(4;14) (70).

2.3.4 RTK class V (VEGF receptor family)

The VEGFR family comprises 3 tyrosine kinase receptors, VEGFR-1 (flt1), VEGFR-2 (KDR/flk-1) and VEGFR-3 (flt4), all of which have seven Ig-like extracellular domains (Figure 3). The expression of these receptors in different tissues and cell lines suggests that each play a unique physiological role. Interestingly, leukemic blasts have been reported to express both receptor and ligand suggesting that an autocrine VEGF-VEGFR system may be pathogenetically relevant in acute leukemia and myelodysplasia (71, 72).

2.3.5 RTK class VII (TRK receptor family)

TRK receptors are expressed on immature erythroblasts (TrkA), eosinophilic metamyelocytes and polymorphonuclear cells (TrkB), as well as promyelocytes, myelocytes and megakaryocytes (TrkC) (73). Reuther and colleagues used retroviral transfer of cDNA libralies to identify novel oncogenes expressed in AML which is a variant of the TrkA protonocogene.

2.3.6 RTK class XI (TIE receptor family)

The TIE receptor tyrosine kinase contains three fibronectin III repeats, a single immunoglobulin-like domain and three EGF-like repeats (Figure 3). High expression of Tie-1, which plays an important role in both angiogenesis and haematopoiesis, has been documented in AML and MDS (71). Tie-2 and angiopoietin-1 are important in the regulation of blood vessel development. Howerver, the recent finding that both receptor and ligand are expressed not only in AML but also MPD, suggesting an involvement in the pathogenesis of MPD (74).

2.3.7 RTK class XIV (RET receptor family)

The RET receptor family has a structure consisting of cadherin-like and cysteine-rich repeats in the extracellular region (Figure 3). RET mutations have been associated with multiple endocrine neoplasia type 2 (MEN2) and the congenital absence of exteric ganglia, or Hirschsprung disease.

2.4 Signaling pathway of RTKs

Knowledge of the cascade of biochemical events triggered by ligand stimulation of tyrosine kinase receptors has increased rapidly in recent years and provides further evidence of the importance of these signaling pathways in cancer. In order to effectively coordinate signaling cascades, nature has created a variety of molecules known as adaptor and scaffolding proteins (75). These proteins play an integral role in intracellular signaling by both recruiting various proteins to specific locations and by assembling networks of proteins particular to a cascade. Adaptor proteins, through protein-protein interactions via specific motifs, provide a link between molecules of a signaling cascade and proteins such as RTKs. One such adaptor, Grb2, is important in the activation of the small G-protein Ras and will be discussed later. These adaptor proteins often contain a variety of motifs that mediate protein-protein interactions. Src homology 2 (SH2) domains are protein motifs that bind to specific phosphorylated tyrosine containing sequences, dictating particular binding partners. SH3 domains recognize and bind to proline-rich sequences in target proteins (76). Thus, in the case of an adaptor protein such as Grb2, which contains both SH2 and SH3 sequences, an adaptor protein can bring a cytoplasmic protein via its SH3 domain to an activated RTK via an SH2 domain binding to phosphorylated tyrosine residues of the receptor.

Another form of adapter, a docking protein, provides multiple binding sites on which effector molecules can attach, thereby expanding the magnitude of responses from an activated RTK. One such docking protein, IRS-1, is a substrate of the IR, which has eighteen possible tyrosine phosphorylation sites (77). It also contains two other important domains: a pleckstrin homology domain (PH) that binds to specific phosphoinositides and a phosphotyrosine-binding (PTB) domain that, like the SH2 domains, binds to phosphorylated tyrosine-containing sequences. These two domains are believed to properly position IRS-1 adjacent to the receptor via the PH domain binding to the plasma membrane and the PTB domain binding to phospho-Tyr of the IR. Proteins that attach to the phosphorylated tyrosine residues phosphorylated in response to IR activation include PI3-K, Shp-2, Nck, and Grb-2 (78).

Another concept that has recently surfaced in mammalian signaling is the importance of so-called scaffolding proteins in signaling cascades. This idea is not

new, and, in fact, it has been known for some time that these proteins exist in the yeast *Saccharomyces cerevesiae*. Scaffolding proteins allow the formation of multienzyme complexes that are involved in a particular cascade. These are important for two reasons. The first is that the activation of a signaling cascade by a growth factor is an extremely rapid process and is not likely to occur as a result of two proteins complexing together by randomly floating in the intracellular milieu until they happen to come in contact with each other. Scaffolding proteins ensure the close proximity of the necessary components. The second reason is that several enzymatic components of a particular signaling cascade may be shared, although the substrates of each may differ. Thus, scaffolding proteins ensure the proper routing of signals by preventing unwanted crosstalks between pathways.

The PDGF system has served as the prototype for identification of the components of signaling cascades. Certain molecules become physically associated and/or phosphorylated by the activated PDGF receptor kinase. Those identified to date include phospholipase C (PLC)-g (79), phosphatidylinositol-3′-kinase (PI-3-K) regulatory subunit (p85) (80), NCK, the phosphatase SHP-2 (81), Grb2 (82), CRK (83), *RAS*, p21, GTPase-activating protein (GAP) (84), and *SRC* and *SRC*-like tyrosine kinases (85). Many of these molecules contain SH2 or SH-3 domains.

PLC-g is one of several PLC isoforms and is involved in the generation of two important second messengers, inositol triphosphate and diacylglycerol (86). The former causes release of stored intracellular calcium and the latter activates protein kinase C (PKC). These second messengers appear rapidly in cells following stimulation by growth factors such as PDGF. The relative increase in their synthesis in vivo correlates reasonably well with the ability of a particular receptor kinase to induce tyrosine phosphorylation of PLC-g (79). In combination with evidence that tyrosine-phosphorylated PLC-g exhibits increased catalytic activity in vitro, it seems very likely that receptor-induced tyrosine phosphorylation activates this enzyme. The actions of a number of tumor promoters are thought to be mediated by PKC. Moreover, PKC overexpression or gene alteration has been reported to increase cell proliferation in culture (87).

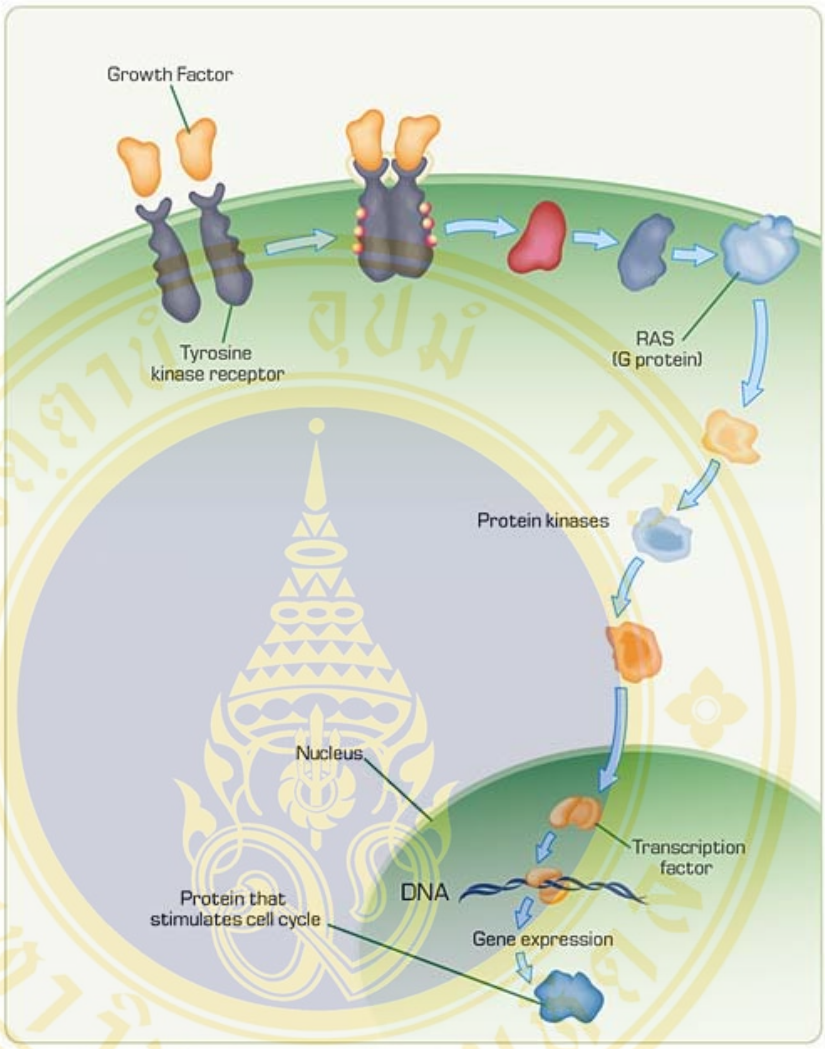

Figure 4 Signaling pathway of RTKs. When a ligand binds to its receptor, the receptor is activated and dimerizes. The signal from the receptor is mediated by various proteins to the nucleus of the cell and initiates transcription of a gene. **(**Figure from www.learner.org/channel/courses/ biology/archive/images/ 1901_d.html)

2.5 Regulation of RTKs by autoinhibition

The physiological regulation of RTKs is a key to understand the mechanisms causing their oncogenic activation (88). Signalling by RTKs requires ligand-induced receptor oligomerization, which results in tyrosine autophosphorylation of the receptor subunits (89). This both activates catalytic activity and generates phosphorylated tyrosine residues that mediate the specific binding of cytoplasmic signaling proteins

containing Src homology-2 (SH2) and protein tyrosine binding (PTB) domains. Crystal structures of the inactive forms of the core cytoplasmic kinase domains of the insulin receptor (INSR), fibroblast growth factor receptor-1 (FGFR-1) and Flk1 tyrosine kinases have provided a molecular understanding of the tight control of catalytic activity resulting from a *cis* inhibition/*trans* activation mechanism (90).

In both the unstimulated FGFR1 and VEGFR2, the activation loop occludes substrate tyrosine binding to the active site, whereas in the unstimulated INSR, Tyr1162 in the activation is bound in *cis* in the active site to prevent substrate access, and the beginning of the activation loop occludes ATP binding. In response to ligand stimulation of RTKs, one of several of the tyrosine residues in the activation loop is phosphorylated in *trans* by the dimeric receptor partner. This leads to repositioning of the activation loop away from the active sites, allowing substrate (and in the case of the INSR, ATP) access. Equilibrium exists between inactive and active loop conformations of the unphosphorylated RTKs in solution (91). This equilibrium enables *cis* inhibition, yet allows phosphorylation in *trans* between ligand-induced receptor dimmers. The importance of this is supported by several examples of oncogemic point mutations in the activation loop the cause constitutive RTK activation.

Evidence indicates that RTK dimerization by itself is not always sufficient for kinase activation. There seems to be an addition requirement for ligand-induced conformational switches, ensuring that the catalytic domains are juxtaposed in a proper configuration to enable phosphorylation in *trans* between receptor subunits (88). Accordingly, other regions of RTKs can have autoinhibitory functions in unstimulated cells for instance, in Tek (TKE2), the carboxy-terminal tail partially occludes the substrate tyrosine-binding site (92). Autophosphorylation of the tail region exposes its phosphotyrosine residues for substrate binding, as well as the substrate-binding site in the kinase. In addition, for some RTKs, including PDGFR, SCFR, CSF1-R, EphR and INSR, the juxtamembrane region has been implicated in autoinhibition. Hence, autophosphorylation of one or two homologous juxtamembrane tyrosine residues in several of these receptors is required for full kinase activation, and mutation to phenylalanine significantly reduces kinase activation (93).

Autophosphorylation of these residues seems to serve a dual function: it relieves the inhibitory conformation enabling full kinase activation and at the same time creates binding sites for numerous SH2-containing signaling molecules, such as Src, RasGAP, SHP-1, SHEP-1 and PI3K. Consistent with a repressive function of the juxtamembrane region, substitution of a Val residue just amino-terminal to the regulatory tyrosine residues in the PDGFR-beta results in constitutive kinase activation (94). In addition, numerous oncogenic mutations in human Kit are located either at the N- or C-terminal to the two tyrosines, and internal gene duplications in the juxtamembrane region of *FLT3* result in constitutive kinase activity (95, 96). The crystal structure of the EphB2 kinase domain has revealed a possible mechanism for the inhibition by its juxtamembrane region. The unphosphorylated juxtamembrane region impinges on the C-alpha helix in the N-terminal kinase lobe and other regions of the kinases, resulting in catalytic repression. The structure also suggests how phosphorylation relives repression by causing dissociation of the juxtamembrane region.

2.6 Class III of RTKs and leukemogenesis

Class III RTKs are characterized by five immunoglobulin (Ig) –like domains in the extracellular region, a single transmembrane domain (TM) a juxtamembrane domain (JM), two intacellular kinase domains (TK1 and TK2) divided by a kinase insert domain (KI), and a C-terminal domain (97). This class including c-fms (98), c-kit, FLT3 (99) and PDGFR beta (100) plays an important role in normal haematopoiesis. c-fms is the receptor for the macrophage-osteoclast lineage(101). FLT3 and c-kit are both required for the survival, proliferation and differentiation of hematopoietic progenitor cells, while c-kit is also important for the growth of mast cells, melanocytes, primordial germ cells and interstitial cells of Cajal (9, 102). The hematopoietic functions of PDGFR beta are less well defined, although the receptor and its ligand probably play a significant role in megakaryocytopoiesis (103).

The chromosome location and genomic structure of the class III RTKs suggests a close evolutionary relationship. The c-kit and PDGFR alpha genes, for example, are located in tandem on chromosome subregion 4q11-q13 (104, 105), while PDGFR beta gene lays approximately 350 base pairs upstream of the major

transcription site of the c-fms gene on chromosome segment 5q31-q33 (106, 107). The human FLT3 gene maps to 13q12 (108) and is closely linked, in a head to tail fasion, to FLT1, a class V RTK.

Many studies showed evidence for the involvement between this class III RTK and leukemogenesis so the following part gives the detail of several members of this class for a better understanding of their role in leukemogenesis.

2.6.1 C-KIT

Human c-kit (CD117), the cellular counterpart of v-kit derived from the Hardy-Zuckerman 4-felline sarcoma virus encodes a glycoprotein receptor that binds stem cell factor (SCF). Immunoprecipitation shows two proteins of approximately 140 kDa and 155 kDa, the larger presumably resulting from glycosylational processing of the smaller protein. c-kit and its ligands play role in normal haematopoiesis, as evidenced by naturally occurring murine mutations at the SI locus, which encodes SCF, as well as in the c-kit receptor itself. These mutations result in varying degrees of macrocytic anaemia and a loss of mast cells, in addition to deficiencies in gametogenesis and melanogenesis (102).

c-kit is downregulated during maturation of all haematopoietic lineages except mast cells, which retain a high level of expression (109). A number of early observations suggested a role for c-kit in leukemogenesis. c-kit, for example, is expressed by myeloblasts in approximately 60-80% of AML patients as assessed by either surface immunostaining or expression of c-kit messenger RNA (110). Moreover, many findings strongly suggested that c-kit mutations might be involved in human mast cell malignancies, AML and Sinonasal (NK/T cell) lymphoma (111-113).

2.6.2 FLT3

FLT3 (fms-like tyrosine kinase, CD135) is expressed in multipotential haematopoietic stem cells and progenitors, suggesting a critical role in stem cell development and differentiation (35, 108). Data from a number of groups have suggested a pathogenic role for FLT3 in AML and other hematologic malignancies, FLT3 mutations commonly found in AML. The details about the relationship between FLT3 and leukemia will be discussed in the next part.

2.6.3 PDGFRβ

PDGFRβ, a 170-190 kDa single transmembrane glycoprotein (CD140b), is predominantly expressed on fibroblasts, smooth muscle cell, glial cells and chrondrocytes. Its ligand plays a fundamental role in wound healing. PDGFRβ, however, has also been identified on a variety of haematopoietic cells, including B and T lymphocyte, NK cells, cultured monocytes, HL60 myelomonocytic cells, platelets and megakaryocytes (114). The concept that PDGFRβ mutations could be leukemogenic was confirmed by Golub et al in 1994 (115), who reported that a rare, but recurring, cytogenetic abnormality in atypical chronic myeloid leukemia, namely t(5;12)(q31:p13), results in a TEL/PDGFRβ fusion gene. As a result, the NH_2-terminus of the receptor is replaced by the first 154 amino acids of the transcription factor TEL, which contains a putative helix-loop-helix (HLH) domain, a motif that enables the fusion receptor to multimerize in the absence of ligand. Many reports of additional chromosomal translocations involving PDGFRβ in patients with MPD also supported and strengthen the pathogenetic association between deregulated tyrosine kinase and myeloid leukemia.

2.6.4 C-FMS

The human *fms* proto-oncogene (c-fms, CD115), located on chromosome 5 and band 5q33.3, encodes a 972 amino acid transmembrane glycoprotein which functions as the receptor of the monocyte colony-stimulating factor (M-CSF or CSF-1). Early studies suggested a leukemogenic role for c-fms. Overexpression of c-fms in mice, for example, leads to the development of myeloblastic leukemia. In addition, studies using hybridization to allele-specific oligonucleotide (ASO) probes identified point mutation in approximately 18% of AML and 15% of MDS patients (116), with codon 969 being more frequently involved than codon 301. Mutations were more common in leukemias characterized by monocytic differentiation, namely acute myelomonocytic leukemia (M4-AML) and chronic myelomonocytic leukemia (CMML). AML1-ETO fusion protein also upregulated c-fms expression by acting on the receptor's promoter, an effect that may explain the high c-fms expression in patients with AML-M2 t(8;21).

3. FMS-like tyrosine kinase 3 (FLT3)

3.1 Structure and function of FLT3

Murine FLT3 was cloned independently by two groups using testis and stem cell fraction of fetal liver as an FMS-homologous kinase, and then a human homologue was cloned (8, 108, 117-119). The FLT3 gene is located on chromosome 13 at q12 and contains 24 exons (formerly 21 exons) (120, 121). FLT3 has two forms; a 158-160 kDa membrane-bound form which has a N-linked glycosylation, and a cytoplasmic 130-143 kDa form that is not glycosylated. FLT3 is predominantly expressed on hematopoietic progenitor cells in bone marrow, thymus and lymph nodes, but is also found in the placenta, brain and gonad. The ligand to FLT3 (FL) is expressed as a membrane-bound and soluble form mainly by bone marrow (BM) stroma cells (122). FLT3 binds to FL and results in dimerization of the receptor, autophosphorylation and the subsequent phosphorylation of cytoplasmic substrates that are involved in signaling pathways regulating the proliferation and differentiation of immature hematopoietic cells. Whereas FL alone shows little proliferation activity in itself, it synergistically enhances the expansion of progenitor cells together with stem-cell factor (the ligand of c-kit) or other cytokines. Furthermore, FL induces the adhesion of hematopoietic progenitor cells to stroma cells via VLA-4 and VLA-5, which play an important role in hematopoiesis (122, 123). The functional role of FLT3 expressed in the central nervous system remains unknown. More convincing evidence of a role for FLT3 has been obtained from FLT3-knockout mice. These animals are born healthy with normal peripheral blood counts, but have reduced numbers of early B-cell precursors in bone marrow. A defect of primitive cells was shown by long-term competitive repopulation assays, indicating a reduced ability to reconstitute B-cell, T-cell and myeloid lineages when transplanted into irradiated mice. Mice deficient in both FLT3 and c-KIT exhibited a more severe phenotype characterized by pancytopenia, suggesting that FLT3 and c-KIT play similar but distinct roles both in multipotent stem cells and in lymphoid differentiation (102). Similarly, mice lacking FL have significantly reduced cellularity in a number of leukocytes, and myeloid and lymphoid progenitors of the bone marrow and reduced numbers of dendritic cells and natural killer (NK) cells in the lymphoid organs, but the phenotype improved with the

administration of FL. On the other hand, overexpression of FL in transgenic mice leads to leukemia development with a long latency period (124).

3.2 FLT3 mutations

3.2.1 FLT3 with internal tandem duplication (ITD)

The first FLT3 mutations to be identified were serendipitously detected during an investigation into the incidence and distribution of FLT3 mRNA in samples from adult AML and childhood ALL patients in Japan (125). Unexpectedly long fragments were detected in the polymerase chain reaction (PCR) products of the JM domain in five out of 30 AML patients. They were also found using genomic DNA from the same patients, excluding the possibility of aberrant alternative splicing. Further analysis showed that they all contained a tandemly duplicated sequence, sometimes with insertion of additional nucleotides. The duplicated region was variable in both size and location in different individuals but always fell within the JM domain encoded by exons 14 and 15 (previously 11 and 12). The resulting transcripts were always in frame and would therefore be expected to produce functional FLT3 chains. Since that first description, numerous other studies have confirmed and extended these findings to the extent that FLT3-ITDs are currently the most frequent single mutation described in AML, with a reported incidence between 13.2% and 32% in adult patients. They have also been detected in 3% of patients with MDS and occasional patients with ALL (126, 127), although some of the latter patients had biphenotypic characteristics. They have not been found in patients with CML, CLL, non-Hodgkin's lymphoma or multiple myelom, or in normal individuals (128, 129).

Preliminary in vitro analysis of FLT3-ITDs transfected into Cos7 cells showed that they induced ligand-independent receptor dimerization and phosphorylation, irrespective of the location and length of the ITD, and led to phosphorylation of wild-type (WT) FLT3 expressed in the same cells (10). They have been shown to confer growth factor independence on factor-dependent cell lines such as Ba/F3 and 32D cells, and to induce constitutive activation of downstream signalling molecules such as signal transducer and activation of transcription 5 (STAT5), mitogen-activated protein kinase (MAP kinase), Akt, Src homology 2 domain-

containing (SHC) transforming protein 1, Cbl, Vav and SH2-containing protein tyrosine phosphatase 2 (SHP2) (11, 130-132).

A striking feature of the FLT3-ITDs identified in AML patients is their diversity in both size and location. In general, the length of the duplication varies between 12 and 204 bp, but it has been reported to be as short as 3 bp and as long as >400 bp. Most ITDs occur at the 5' end of the JM domain, in exon 14, and the TK1 domain is minimally involved, but they differ in the precise sequence duplicated and its starting point. Several inserted sequences of unknown origin varying between 9 and 36 bp have also been detected (10, 15, 133). There is no sequence that is common to all reported duplications, but the involved region does contain a tyrosine-rich stretch of sequence, and most ITDs include at least one of the tyrosines 589, 591, 597 or 599, which form part of the motifs YFYV and YEYDLK. Current models suggest that the JM domain has a negative regulatory role that is disrupted by the ITD elongation. In the wild-type receptor, the JM domain takes up an A-helical conformation, which blocks activation of the kinase and may inhibit self-dimerization (Figure 5). Ligand binding overcomes the inhibitory effect by inducing a conformational change and/or phosphorylation of key tyrosine residues. The ITDs may therefore prevent the protective association between the JM domain and kinase, exposing the latter to constitutive activation. They may further allow recruitment of molecules that could stabilize this conformation or alter downstream signalling. The in vivo tumorigenic potential of a FLT3-ITD has been demonstrated by injection of 32D cells carrying a FLT3-ITD into syngeneic mice, which led to the rapid development of a leukaemia-like disease (11). Furthermore, AML cells with an ITD showed an increased ability to repopulate bone marrow in non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice (134). However, although transplantation of bone marrow cells retrovirally transduced with FLT3-ITDs into recipient mice led to an oligoclonal myeloproliferative disorder, it was insufficient to cause leukaemia (135). This indicates the requirement for additional cooperating mutations for a fully transformed phenotype in this model. Further evidence for this co-operation has come from transplantation of cells carrying FLT3-ITD into PML/RARa transgenic mice, which considerably shortened the latency and increased the penetrance for developing an APL-like disease (136). It can be hypothesized that, in such murine models, only

'two hits' are required to generate leukaemia, the mutation in the transcription factor (e.g. PML/RARa) producing a block in differentiation, and the mutant growth factor receptor providing a proliferative or survival signal (137).

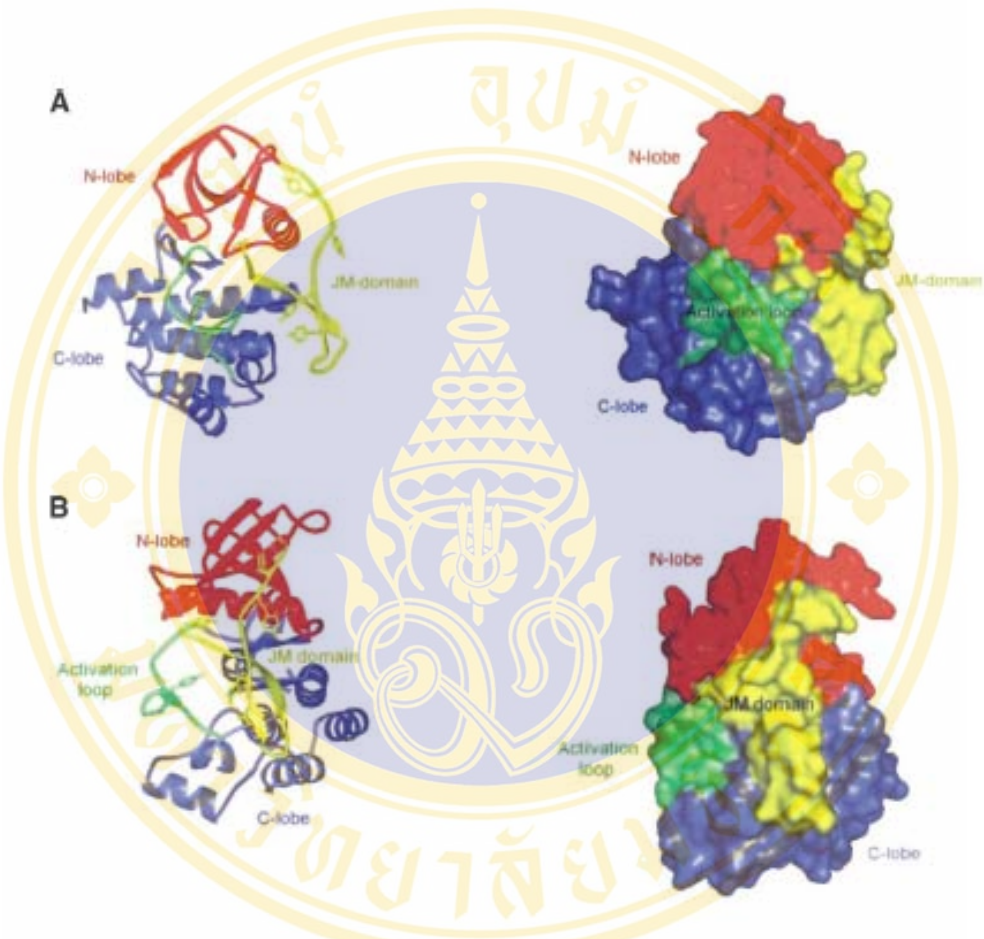

Figure 5 Structure of autoinhibited FLT3 (A) Ribbon and surface diagrams of FLT3 showing the spatial arrangement of the various structural elements of the molecule. The N-terminal kinase domain (red) and the C-terminal kinase domain (blue) comprise the standard kinase fold. The activation loop (green) is folded up between the two kinase domains. The JM domain (yellow) nearly spans the length of molecule. All tyrosines in the JM domain and the activation loop are displayed as "stick" representations. (B) Same as (A), except the molecule is rotated 90° clockwise when viewed down the vertical axis. (Griffith *et al*, 2004) (138)

3.2.2 Mutation in the tyrosine kinase domain (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 and Abu-Duhier et al in the year 2001 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. The complete deletions of I836, insertion of nucleotides and complex changes have also been detected, but the sequence always remains in frame. The incidence varies between 6.4% and 7.7% in unselected adult AML patients. Occasional cases have been reported in ALL (2.8%) and MDS (3.4%) (14). The mutations were found to cause constitutive tyrosine phosphorylation of the receptor when transfected into Cos 7 cells, and to confer IL-3-independent growth on 32D cells, indicating their gain-of-function property (14). The amino acids are part of the activation loop that blocks the access of ATP and the substrate to the kinase domain when the receptor is in an inactive state. Ligand-induced activation leads to phosphorylation within the loop causing it to take up an active configuration and allow kinase activity. The TKD mutations are thought to mimic the latter by interfering with the inhibitory effect of the loop, resulting in constitutive kinase activation. Their effect is therefore similar to that of FLT3-ITD mutations in disrupting the autoinhibitory mechanisms that normally protect the cell from unregulated signalling through FLT3. However, possible differences in the functional consequences of the various TKD mutations have not been investigated. A further constitutively activating FLT3 mutation in exon 20 has been reported recently in 2002 by Spiekermann *et al* (16), who detected an additional 6 bp leading to the insertion of glycine and serine between amino acids 840 and 841 in two out of 359 (0.5%) de novo AML patients. Functional analysis demonstrated that the mutant receptor was constitutively phosphorylated and induced factor independence in Ba／F3 cells. Like the D835／I836 mutations, it is probable that this mutation also interferes with the inhibitory role of the activation loop.

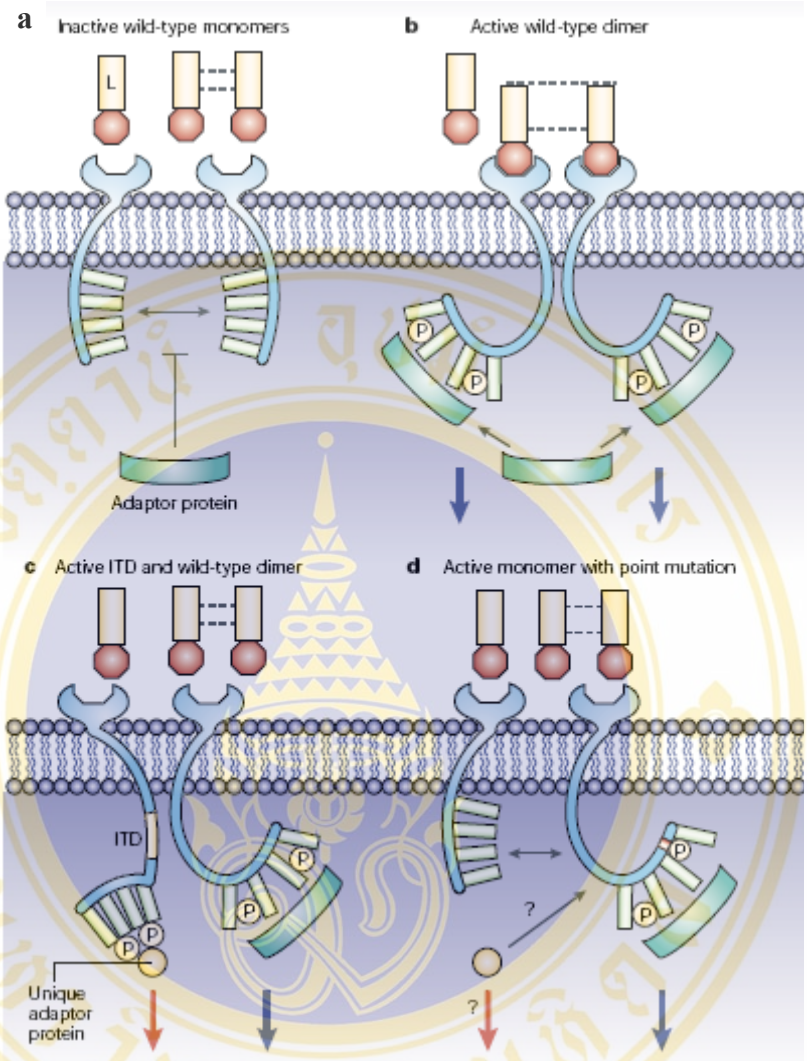

Figure 6 A comparison of the possible modes of activation of wild-type FLT3 and mutant FLT3. **a** | Without ligand (L), receptors remain mostly in the inactivated monomeric form. The conformation of the monomeric receptor probably involves repulsive forces that prevent dimerization and possibly block adaptor proteins (green) from docking. **b** | After ligand binding, conformational changes in FLT3 promote dimerization, phosphorylation (P) and adaptor-protein docking, transmitting signals to downstream effectors (green arrow). **c** | ITDs probably promote ligand-independent dimerization and activation of FLT3 through changing the conformation of the expressed receptor. Experiments indicate that ITD-containing FLT3 might activate both similar (blue) and unique (red) pathways compared with wild-type FLT3. The conformational changes associated with ITDs might change the structure of the receptor such that unique adaptor proteins can now dock. **d** | Missense mutations in TKD2 might also promote ligand-independent activation. It is unknown if dimerization is required or if the downstream effectors are similar to those activated through the wild-type cascade. (Stirewalt *et al*, 2003) (139)

3.2.3 Mechanism of FLT3-induced leukemia

3.2.3.1 Internal tandem duplication

The exact pathway by which activating mutations of FLT3 lead to leukemia is not completely clear. However, experimental data suggests that signaling through both FLT3 may contribute through enhancing proliferation, inhibiting apoptosis and blocking differentiation.

Several evidence show that the anti-apoptotic pathway involving PI3-kinase and Akt activation is involved in signaling through both *FLT3*-wild type and ITD receptors. The proliferative pathway involving RAS and MAP kinase has also been shown to be activated in FLT3 signaling. STAT5 is the one signaling protein whose activation has been convincingly shown to differ between wild-type *FLT3* and constitutively activated signaling. Ba/F3 cells transfected with constitutively activated *TEL-FLT3* fusion product showed constitutive activation of STAT5, as did Ba/F3 and 32D cells transfected with *FLT3*-ITD (130, 132, 140). *FLT3* wild-type transfectants, on the other hand did not show constitutive activation of STAT5.

Another potential mechanism by which *FLT3*-ITD may contribute to the leukemic phenotype is inhibition of phosphatase activity. The effects of the *FLT3*-ITD on activation of tyrosine kinase signaling may be enhanced by the inhibition of cellular phosphatases such as SHP-1, which could lead to amplification of the proliferative, andti-apoptotic and differentiation-blocking effects. Data from one laboratory has shown that Ba/F3 cells transfected with *FLT3*-ITD suppressed SHP-1 expression, while those transfected with wild-type *FLT3* did not (141).

3.2.3.2 Activation loop mutation

The biological significance of activation loop mutations is less clear. When 32D and Ba/F3 cells transfected with the Asp835Tyr mutation, constitutive receptor phosphorylation and IL-3 independent proliferation occurred (14, 142). On the other hand, point mutations do not appear to affect prognosis to the same degree as ITD, at least in adult AML (15, 133). It may be that there is a qualitative ofrquantitative difference in FLT3 signaling induced by the ITD and the point

mutation, even though both clearly result in constitutive kinase domain activation and autophosphorylation.

3.2.3.3 Overexpression/activation of wild-type FLT3

The significance of overexpression and activation of wild-type FLT3 in leukemias is also not clear. Ba/F3 cells overexpressing wild type FLT3 were not factor-independent *in vitro*, but caused leukemia when injected in Balb/c mice, perhaps as a result of ligand expression by the mice (140). Balb/c mice transplanted with bone marrow transduced with a constitutively expressed FL gene develop leukemia, though with a long latency (124). These experiments suggest that autocrine and paracrine signaling through wild-type receptors may play a role in leukemogenesis. Several human leukemia-derived cell lines, such as EOL-1 and REH, demonstrated constitutive activation of FLT3 without identifiable FLT3 activation mutations.

3.3 FLT3 inhibitors

The prognostic significance of FLT3 activating mutations in AML adds to the credentials of this receptor as a potential therapeutic target. While the recent eliminary clinical success of the ABL tyrosine kinase inhibitor imatinib mesylate in CML has provided an important precedent, the role of FLT3/ITD mutations in AML is perhaps more analogous to that of HER2 in breast cancer, or to that of the BCR-ABL mutation in Philadelphia chromosome positive ALL (143, 144). In each of these examples, like FLT3 in AML, the targeted gene product is but one of several alterations that causes the malignancy. HER2, a member of the ErbB receptor tyrosine kinase family, is amplified or overexpressed in 20–30% of patients with breast cancer. These patients have been recognized as having a poor prognosis. Treatment of this subset of breast cancer patients with trastuzimab, a monoclonal antibody directed against HER2, leads to improved survival, but not cures. Likewise, the presence of BCR-ABL is an adverse prognostic factor in ALL, and the use of imatinib mesylate in the subset of ALL patients who harbor this mutation has resulted in favorable clinical responses. Translational research efforts have thus validated HER2 and the ABL kinase as therapeutic targets in these situations. However, inhibition of one ‘hit’

among the several that are likely responsible for these malignancies is insufficient to cure them. The first FLT3 inhibitors to be identified were the tyrphostins AG1295 and AG1296. These compounds, originally characterized as PDGF-R inhibitors, inhibit FLT3 autophosphorylation in vitro (each with an IC50 of 300 nM), and are cytotoxic to FLT3-dependent cell lines at doses that correspond to the inhibition of FLT3 autophosphorylation. Furthermore, AG1295 was selectively cytotoxic to primary AML blasts harboring FLT3/ITD mutations, a finding that further validates FLT3 as a therapeutic target. Another compound, CEP-701, has been shown to inhibit very potently and more selectively FLT3 autophosphorylation in vitro and in vivo (IC50 of 2–3 nM for FLT3 compared with an IC50 of 4500 nM for KIT), and, like AG1295, is preferentially cytotoxic to leukemia cells harboring constitutively activated FLT3 (145).

A number of additional small-molecule tyrosine kinase inhibitors with activity against FLT3 have now been identified. These FLT3 inhibitors encompass a variety of chemical classes, but all are heterocyclic compounds containing a structural mimic of the purine component of ATP. While direct structural analysis of FLT3 is not yet available, analysis of the crystal structure data for a variety of other kinases bound to different small-molecule inhibitors allows some deductions to be made regarding the structure activity relationships of FLT3 inhibitors. Even though these compounds are all likely to fit into the ATP binding pocket of the receptor, some may do so via an induced fit mechanism (like the indolocarbazole staurosporine appears to do when binding to CSK), while others bind in a lock-and-key manner (such as imatinib binding to ABL). Inferring from the situation with other drugs, FLT3 inhibitors may bind to the active, inactive, or transitional state of FLT3. Selectivity for FLT3 may be greatly influenced by differences in single amino-acid residues between different receptors. For example, FLT3 is normally insensitive to inhibition by imatinib mesylate (IC50 43 mM). However, substitution of Phe-691 with threonine renders the receptor susceptible to the drug (IC50 0.1–0.3 mM). FLT3 inhibitors cause cell cycle arrest, inhibit proliferation, and induce apoptosis in a variety of FLT3-dependent cell lines. In a number of different studies, the FLT3 inhibitors as discussed aboved have been shown to be cytotoxic to the human leukemia-derived cell lines MV4-11, MOLM-13, MOLM-14, EOL-1, and SEMK2, all of which express constitutively

activated FLT3. They likewise induce apoptosis in murine 32D and Ba/F3 cells transfected with *FLT3*-ITD constructs, and are selectively cytotoxic to primary AML blasts harboring *FLT3*-ITD mutations. Finally, they lead to prolonged survival in a number of murine *FLT3*-ITD leukemia models, including Ba/F3-ITD cells injected into syngeneic Balb/c mice, MV4-11 and SEMK2 xenografts, and an *FLT3*-ITD bone marrow transplant model (145).

There is also the question of which patients will potentially benefit from FLT3 inhibitors. As most AML cells express FLT3, it is not surprising that some leukemia samples lacking activation mutations still respond in vitro to FLT3 inhibition. On the other hand, a minority of primary patient samples harboring *FLT3*-ITD mutations fail to respond to FLT3 inhibition (15–20%). In this regard, the development of laboratory methods for reliably predicting clinical response to FLT3 inhibition is important (145).

3.4 Techniques for screening of *FLT3* mutations

The traditional approach for diagnostic screening of *FLT3* mutations in AML patients involves polymerase chain reaction (PCR) followed by gel electrophoresis or RFLP. However, the other approaches are also performed to increase sensitivity and more advantage. These molecular techniques include single strand conformational polymorphism (SSCP), conformational sensative gel electrophoresis (CSGE), capillary electrophoresis (CE), denaturing high performance liquid chromatography (D-HPLC) and non-isotopic RNase cleavage assay. All of these approaches would be done after standard or multiplex-PCR.

3.4.1 Single strand conformational polymorphism (SSCP)

Single stranded DNA (ss-DNA) has the properties to fold up and form complex structure stabilized by weak intramolecular bonds. The electrophoretic mobility of such structure in nondenaturing conditions will depend not only on their chain length but also on their conformations, which are dictated by the DNA sequence. Single strand conformation polymorphism (SSCP) can be used to detect the sequence variations based on the different mobility of ss-DNA. SSCP are detected by amplifying the DNA samples, denaturing, immediately cooling and loading on a nondenaturing polyacrylamide gel. Primers can be radiolabled, or unlableled products

can be detected by silver stained by silver staining. The mobility patterns of bands depend on details of the conditions such as the charge of molecules, temperature, presence of gel additives, percentage of acrylamide cross-linking, and ionic strength of buffer. Wild type samples must be run, thus difference from the wild type pattern can be noticed. SSCP is very cheap and reasonable sensitive (~80%) for DNA fragments up to 300 bp. In addition, Stirewal *et al* identified the novel missense point mutations in exon 14 of *FLT3* gene by using this SSCP analysis in patients with AML (146).

3.4.2 Conformational sensative gel electrophoresis (CSGE)

Classic methods detect unknown polymorphisms by observing the different electrophoretic migration behaviors of homoduplex versus heteroduplex DNA. Moreover, this technique were used to screen *FLT3* mutations by Abu-Duhier *et al* and identified novel FLT3 Asp835 mutations in adult AML (147).

3.4.3 Capillary electrophoresis

Electrophoresis is a powerful method that was developed long before molecular biology came into existence. The principle behind electrophoresis is that charged molecules will migrate toward the opposite pole and separate from each other based on physical characteristics. For example, SDS-PAGE separates proteins according to their molecular weights. The two limiting factors of traditional electrophoresis were: 1) detection of molecules upon completion of electrophoretic separation and 2) only low voltages could be used to prevent heat damage of the samples. Capillary electrophoresis (CE) solved both of these problems. Because of the capillary tube has a high surface to volume ratio (25-100 μm diameter), it radiates heat readily and thus samples do not over heat. This approach was combined together with multiplex PCR for detection FLT3-ITD and FLT3-D835 mutation in a single assay by two groups, Murphy *et al* and Anasari-Lari *et al*.

3.4.4 Denaturing high performance liquid chromatography (D-HPLC)

D-HPLC is based on the principle that heteroduplex DNA can be distinguished from homoduplex DNA by ion pair reverse phase chromatography under

partially denaturing conditions. D-HPLC allowed high throughput thus at present many studies used this approach to screen novel mutations. The limitation is required the expensive equipment and the results do not reveal position of change. Moreover, Bianshini et al showed this approach is optimized for identification for novel point mutations in the catalytic and regulatory domains of FLT3 receptor (148).

3.4.5 Non-isotopic RNase cleavage assay (NIRCA)

Ribonuclease A cleavage was originally described by Myers *et al* using DNA:RNA hybrids. Sensitivity was reported to be around 60% per strand cleaved. Grange *et al* described improved sensitivity by screening both strands of RNA. A Non-Isotopic RNase Cleavage Assay (NIRCA) has also been described. This assay (commercially available from Ambion) utilized PCR primers with 'phage RNA polymerase promoters so that large quantities of RNA were produced. Cleaved products could be detected on agarose gels and fragments of up to 1 kilobase were analyzed. In principle screening both strands for mismatches might increase the sensitivity to 80 or 90%. Additional RNase enzymes such as RNase 1 and RNase T1 increase the sensitivity of the assay. The commercial kit includes a helix modifying reagent that makes the mismatches more sensitive to cleavage. As a fast and easy screening method for large fragments, this method has much in its favor. The major disadvantages are lack of 100% sensitivity and the need to make primers which include 'phage polymerase promoters. However since large (1 Kb) DNA fragments are often amplified with 'phage promoters as part of the protein truncation test, it may be cost-effective to use those amplimers to produce template for RNase cleavage. Recently, Mutsuno *et al*, identified the novel FLT3 activation loop mutation, N841K, by using this NIRCA technique in AML samples (149).

The additional technique mostly described to increase useful for detection of FLT3 mutation, including increase sensitivity to detect novel mutation and less time to screening this mutation. However, the traditional method still beneficial for screening known mutations in large series of samples as done in many studies and these studies frequently showed the correlation between clinical outcome and/or prognosis with *FLT3* mutations.

CHAPTER IV
MATERIALS AND METHODS

1. Materials

1.1. Subjects and samples

The subjects included in the study were 256 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 85 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.

For normal controls, peripheral blood mononuclear cells from 30 healthy volunteers working at the Faculty of Medicine Siriraj Hospital, Mahidol University were used.

1.2. Oligonucleotide primers

Previously published primers used for PCR amplification and sequencing reaction were 11F, 12R, R5, R6, 17F, 17R, and MR2 (15, 150). Genome sequences and mRNA sequences of *FLT3* gene were obtained from GenBank database under the accession number NT_009799 and NM_004119, respectively.

The relative positions of these primers are shown in Figure 7. The locations of seven primer pairs designed for amplification of *FLT3* gene are shown in Figures 8, 9 and 10. Nucleotide Sequences, numbers, melting temperatures (Tm), and product sizes after amplifications with all primers are listed in Table 3.

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

1.3. Other materials

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

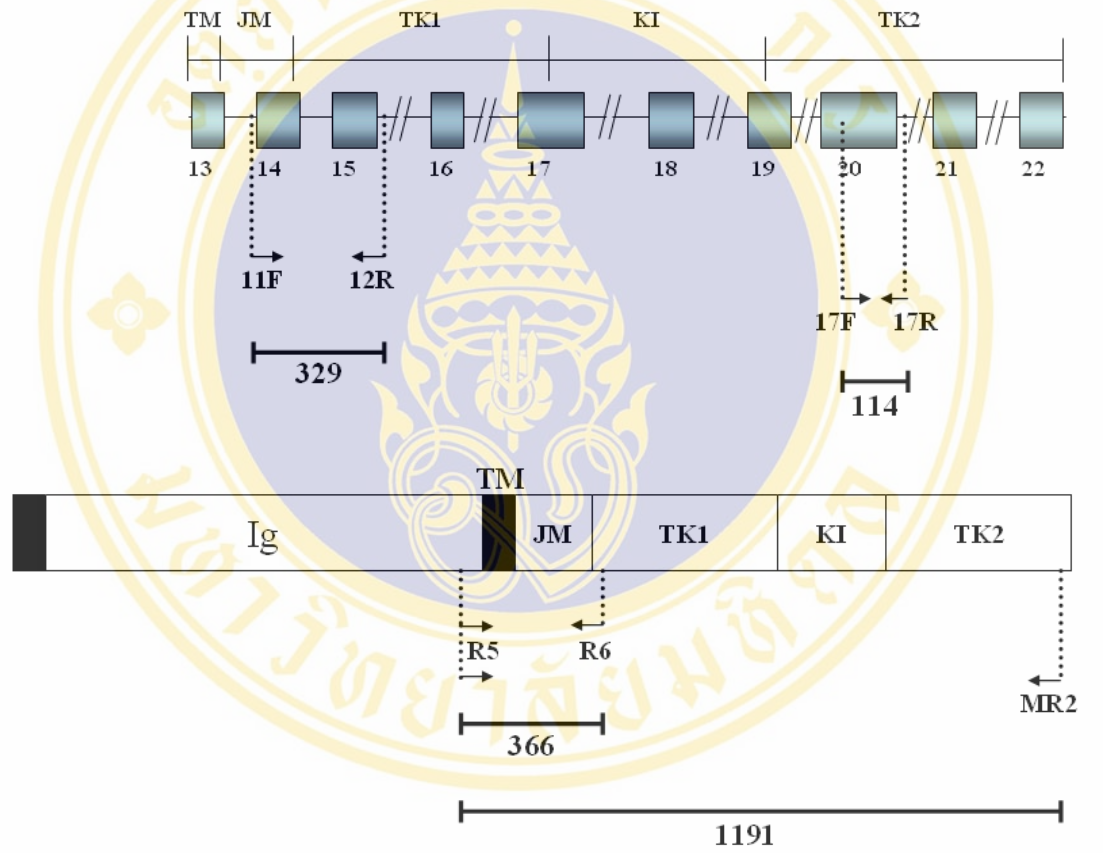

Figure 7 Schematic representation of the *FLT3* gene showing the locations of primers used for amplifications of ITD, TKD and dual mutations. Genomic organization of the downstream part of FLT3 gene, positions of primers and product size are shown in the upper panel. Structure of full-length cDNA of *FLT3* gene and position of primers are shown in the lower panel.

Figure 8 Nucleotide sequence of exons 13 - 15 of *FLT3* gene. Exons are indicated in blocks. Underlined are sequences used to design primers. Arrows indicate 5' to 3' direction of the primers (primers 11F and 12R). Complete sequence of the *FLT3* gene (accession number NT_009799) was retrieved from GenBank database.

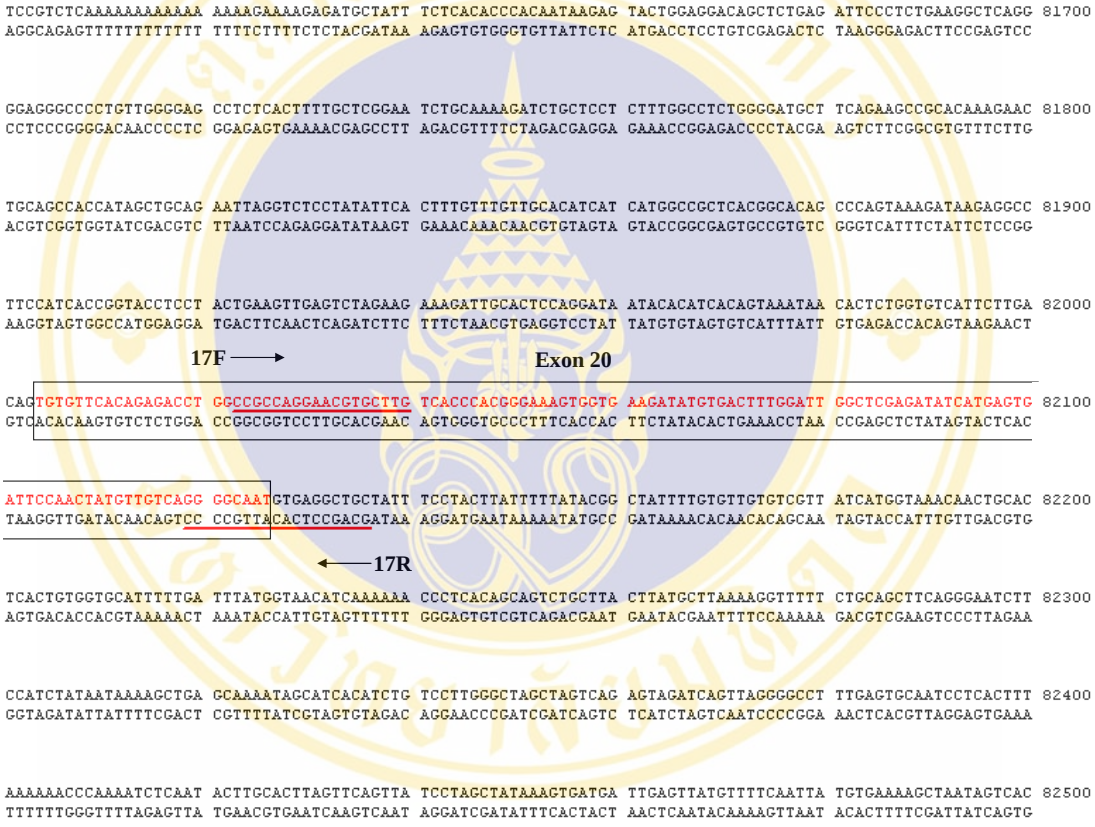

Figure 9 Nucleotide sequence of exon of *FLT3* gene. Exon is indicated in block. Underlined are sequences used to design primers. Arrows indicate 5' to 3' direction of the primers (primers 17F and 17R).

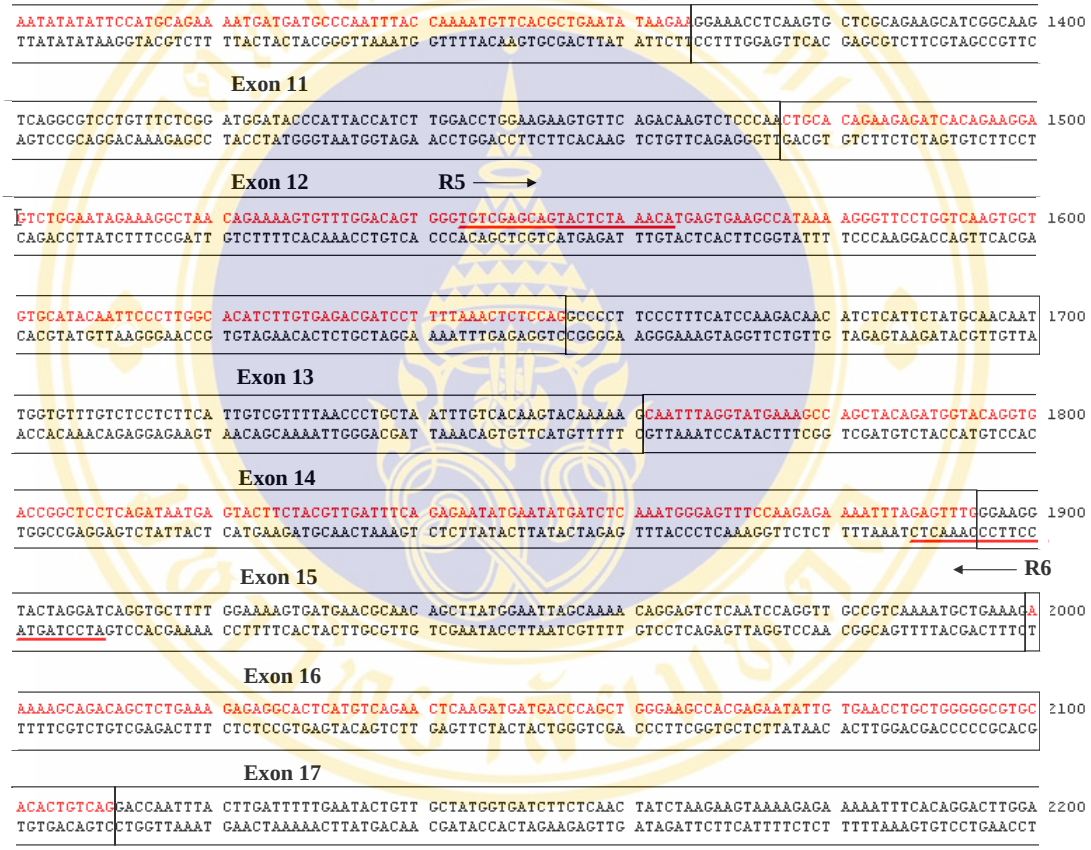

Figure 10 Nucleotide sequence of exons 11 – 17 of *FLT3* mRNA. Arrows indicate 5' to 3' direction of the primers (primers R5 and R6). Complete sequence of the *FLT3* mRNA (accession number NM_004119) was retrieved from GenBank database.

Table 3 Primers used in the amplification of *FLT3* gene.

Fragment	Primer	Nucleotide sequence (5' - 3')	No. of nucleotide	Tm (C°)	Product size (bp)
Ex 14-15	11F	GCAATTTAGGTATGAAAGCCAGC	23	57	329
	12R	CTTTCAGCATTTTGACGGCAACC	23	58	
Ex 20	17F	CCGCCAGGAACGTGCTTG	18	59	114
	17R	GCAGCCTCACATTGCCCC	18	59	
Ex 12-15	R5	TGTCGAGCAGTACTCTAAACA	21	54	366
	R6	ATCCTAGTACCTTCCCAAACTC	22	56	
Ex 12-22	R5	TGTCGAGCAGTACTCTAAACA	21	54	1191
	MR2	GAATGCCAGGGTAAGGATTCACA	23	60	

2. Methods

2.1. Experimental strategy

The experimental steps began by searching for previously published primers for amplification of *FLT3* gene. Mononuclear cells from AML patients were collected for further use in nucleic acid isolation. Genomic DNA and RNA were prepared. PCR and RT-PCR were optimized, and also done in RFLP condition. Each part of *FLT3* gene, including transmembrane (TM), juxtamembrane (JM), and kinase domain region was amplified from DNA and cDNA by PCR and RT-PCR, respectively. The products were screened for mutation by gel electrophoresis or RFLP followed by gel electrophoresis. The aberrant products of *FLT3*-ITD and *EcoR*V undigested products that showed *FLT3*-TKD mutation were analyzed by sequencing to assess the extent of duplication and characterize the nucleotide change. The results of FLT3 genotypes were correlated with immunophenotypes, karyotypes, and clinical data. The experimental strategy is shown in Figure 11.

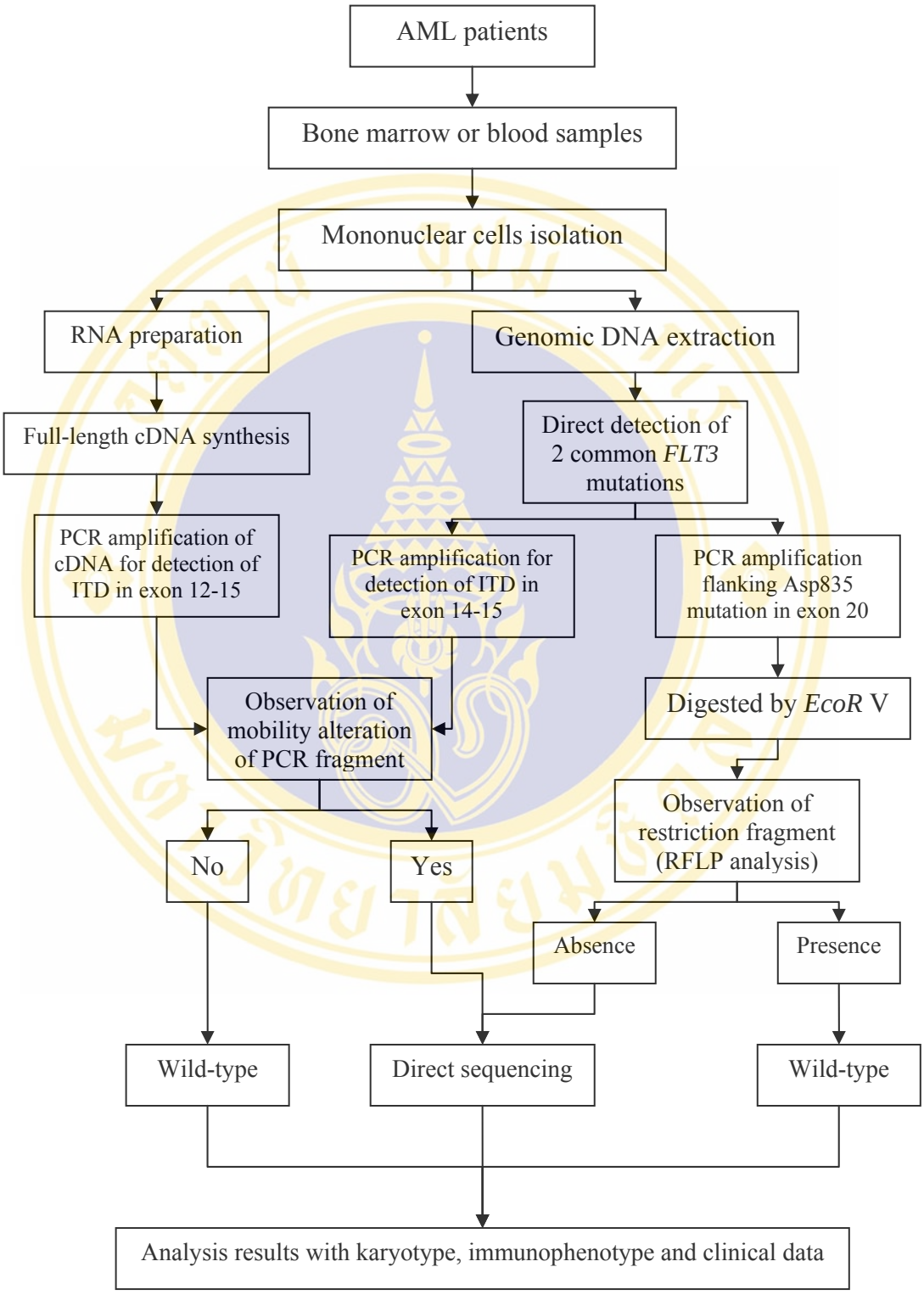

Figure 11 Flow chart of experimental strategy used to study *FLT3* mutations in Thai AML patients.

2.2. Separation of mononuclear cells

Mononuclear cells were separated from heparinized bone marrow or blood samples for nucleic acid preparation. The samples were isolated by Ficoll-hypaque gradient centrifugation. The samples were diluted with three volume of phosphate Buffered Saline (PBS) and layered on Ficoll-hypaque reagent and then centrifuged at 1,000 g for 20 minutes. The mononuclear cells layer was then collected, washed twice with PBS, pelleted by centrifugation and stored in 1.5 ml microtubes until use for nucleic acid preparation

2.3. 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 manufacturer. After isolation of total RNA, the genomic DNA was harvested from the remaining suspension by precipitation followed by a series of washes according to the supplier's recommendations.

The full-length cDNA was amplified from mRNA using the genetically engineered reverse transcriptase which lack RNase H activity, SuperScript™II RT (Invitrogen Corporation, CA, USA) and oligo-dT primer for first strand cDNA synthesis.

2.4. Analysis of *FLT3* internal tandem duplication (ITD) mutation by PCR and RT-PCR

For amplification of exon 14 to exon 15 of *FLT3* gene, PCR reaction was carried out in a 200 µl microtube using a 25 µl PCR mixture which contained 2.5 ml of 10x PCR buffer, 0.5 µl of 50 mM of $MgCl_2$, 0.5 µl of 10 mM of dNTP, 1 µl of 10 pmol/µl of each 11F and 12R primers, 0.125 of 1U/µl of *Taq* DNA polymerase (Immolase, Bioline, Germany), 17.375 ul of sterile distilled water, and 2 µl of 25 ng/µl of DNA template.

In order to confirm the present of *FLT3*-ITD mutation found by DNA analysis and rule out the possibility of abnormal RNA splicing in *FLT3* gene, the cDNA from reverse transcription reaction was also used as a template for PCR with

R5 and R6 primer pairs. The PCR mixtures and amplification method were similar to the PCR reaction as explained above.

PCR was performed for 30 cycles in Thermal Cycler (Perkin Elmer PCR2400, USA). The detail of PCR cycles were as followed (i) denaturation at 94 °C for 1 minute (12 minutes for the first cycle), (ii) annealing at 59 °C for 1 minute, (iii) extension at 72 ° C for 2 minute (10 minutes for the last cycle).

The PCR products were detected by agarose gel electrophoresis. Five microlitres of PCR products were mixed with 1 microlitre of 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 µg/ml ethidium bromide solution and the PCR products were viewed on gel documentation machine.

2.5. Analysis of *FLT3* tyrosine kinase domain (TKD) mutation by PCR-RFLP

Two primers (17F and 17R) 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 PCR buffer, 0.5 µl of 50 mM of $MgCl_2$, 0.5 µl of 10 mM of dNTP, 1 µl of 10 pmol/ul of each 17F and 17R primers, 0.125 of 1U/ul of *Taq* DNA polymerase (Immolase, Bioline, Germany), 17.375 µl of sterile distilled water, and 100 ng of DNA template. PCR was performed for 30 cycles in Thermal Cycler (Perkin Elmer PCR2400, USA). The details of PCR cycles were as followed (i) denaturation at 94 °C for 30 seconds (7 minutes for the first cycle), (ii) annealing at 59 °C for 30 seconds, (iii) extension at 72 ° C for 1 minute (5 minutes for the last cycle).

The PCR products were digested with restriction enzyme *EcoR*V as previously described. The digestion reaction was carried out in 20 µl which contained 1 µl of 5x *EcoR*V buffer, 0.25 µl of 1U/µl *EcoR*V 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, 50 bp DNA ladder was also used as a standard-size marker.

2.6. Analysis of FLT3-Dual mutation by PCR-RFLP

FLT3 gene region of exon 14 to exon 22, including region from JM through TK2 domain was studied by RT-PCR using R5 and MR2 primers. PCR reaction contained 2.5 µl of 10x PCR buffer, 1 µl of 50 mM of $MgCl_2$, 0.5 µl of 10 mM of dNTP, 1.5 µl of 10 pmol/ul of each R5 and MR2 primers, 0.125 of 1U/ul of *Taq* DNA polymerase (Immolase, Bioline, Germany), 14.875 µl of sterile distilled water, and 100 ng of DNA template. PCR was performed for 30 cycles in Thermal Cycler (Perkin Elmer PCR2400, USA). The detail of PCR cycles were as followed (i) denaturation at 94 °C for 30 seconds (95 °C for 7 minutes for the first cycle), (ii) annealing at 63 °C for 30 seconds, (iii) extension at 72 ° C for 1 minute (10 minutes for the last cycle).

The PCR products were digested with restriction enzyme *EcoR*V, as explained above.

2.7. Sequencing of PCR products

2.7.1. Purification of PCR products

The PCR product that showed aberrant pattern of mobility shift in agarose gel electrophoresis and the RFLP products that undigested with *EcoR*V were amplified and purified for sequencing analysis by using QIAquick Gel Extraction kit. (QIAgen, Chatsworth, CA, USA).

For *FLT3*-ITD, the aberrant product was separated by electrophoresis on 3% agarose gel. It was excised with a sharp blade and placed into 15 ml centrifuge tube. The volume of gel was estimated from its weigh and three volume of QG buffer were added to the gel. The gel was then incubated at 50 °C for 10 minutes in a waterbath until gel completely dissolved. One gel volume of isopropanol was added. After that, the mixture was applied to QIAquick column and the column was then placed in a 2 ml collection tube. The column set was spun at 10,000 g for 1 minute, which the PCR products were trapped by the column. The residual agarose was removed from the column by adding 0.5 ml of QG buffer and centrifuged again. The column was washed with 0.75 ml of PE buffer and spun again. The PCR products were eluted from the column by soaking with 30 µl of elution buffer of distilled water for 1 minute and the

products solution was collected into a 1.5 ml microtube by centrifugation at 10,000 g for 1 minute. The purified product was run on agarose gel electrophoresis and its concentration was estimated from band intensity by comparing to standard DNA markers, Φx174 DNA digested with HaeIII.

For *FLT3*-TKD the amplified products from *FLT3*-TKD samples were mixed with five volume of PBS. The mixture was applied to the QIAquick column and the column was then placed in 2 ml collection tube. The column set was spun at 10,000 g for 1 minute, which the PCR products were trapped by the column. After that, the column was washed with 0.75 ml PE buffer and followed by the similar method as explained above.

2.7.2. Cycle sequencing

Nucleotide sequence of the purified PCR product was analyzed by automatic sequencer, ABI-PRISM 310 Genetic Analyzer (PE-Applied Biosystems, USA). Before the analysis, cycle sequencing reaction was performed by using ABI-PRISM BigDye™ Terminators Cycle Sequencing Kit (PE-Applied Biosystems, USA), following the manufacturers protocol.

The reaction mixture was made by mixing 4 µl terminator ready reaction mix, 3.2 pmol primer, 30-40 ng of purified PCR product (as DNA template), and distilled water to a total volume of 20 µl. Using a Thermal Cycler (Perkin Elmer PCR2400, USA), the DNA template in a mixture was amplified for 25 cycles, each of which consisted of 96 °C for 10 second, 50 °C for 5 second, and 60 °C for 4 minutes.

2.7.3. Preparation of cycle sequencing product for loading

The cycle sequencing product was purified from the excessive dye terminators by precipitation with 2 µl of 3 M sodium acetate, pH 4.6, and 50 µl of absolute ethanol, the pellet was rinsed with 250 µl of 70% ethanol and dried in the dark place for 10-15 minutes. The dried pellet was suspended in 25 µl template suppression reagent (TSR), heated at 95 °C for 2 minutes to denature the sequencing product, and chilled on ice. The suspension was then loaded to the ABI-PRISM 310 Genetic Analyzer (PE-Applied Biosystems, USA).

2.7.4. Analysis of DNA sequence

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

2.8. Analysis of novel *FLT3*-deletion mutation by cloning and sequencing

2.8.1. Cloning of *Taq* polymerase-amplified PCR products

One sample that showed a novel *FLT3*-D835Del+I836V mutation was re-amplified and used as a template for cloning. The TOPO® cloning reaction in 6 µl contained 4 µl of fresh PCR product, 1 µl of salt solution, and 1 µl of TOPO® vector. The mixture was then gently mixed and incubated in room temperature for 30 minutes. After that, the reaction was placed on ice and preceded to Transforming One Shot® competent cells.

For each transformation, one vial of competent cells and three selective plates were prepared. The details of this method are as followed, (i) the vial of SOC medium was warmed to room temperature and the selective plates were also warmed at 37 °C for 30 minutes. (ii) Forty microlitres of 40 mg/ml X-gal and 40 µl of 100 mM IPTG were spread on each LB plate and incubated at 37 °C until ready to use.

For One Shot® Chemical transformation, 2 µl of TOPO® cloning reaction from above performing was added into a vial of One Shot® competent *E. coli* and gently mixed. The mixture was then incubated on ice for 5 to 30 minutes. The cells were heat-shocked for 30 seconds at 42 °C without shaking and immediately transferred the tube to ice. After that, 250 µl of room temperature SOC medium was added to the tube. The tube was then tightly capped and shake horizontally (200 rpm) at 37 °C for 1 hour. The reaction mixture was then spread on each pre-warmed selective plate by varied amount of mixture (10 µl, 20 µl, and 30 µl) and incubated overnight at 37 °C. The white or light blue colonies were picked for analysis.

For analysis of positive transformants, the PCR method for *FLT3*-TKD mutation as explained above was performed and followed by *Eco*RV-RFLP. The

colony that showed undigested product of the mutant was then cultured in 5 ml of LB broth and incubated overnight at 37 °C.

2.8.2. Plasmid mini purification

The pellets of bacteria were collected by centrifugation and then re-suspended in 0.3 ml of Buffer P1. The mixture was added with 0.3 ml of Buffer P2 and gently mixed and incubated at room temperature for 5 minutes. After that, 0.3 ml of chilled Buffer P3 was added to the mixture and immediately but gently mixed and placed on ice for 5 minutes. The reaction tube was then centrifuged at maximum speed in a microcentrifuge for 10 minutes and the supernatant was promptly removed and kept for the following step. A QIAGEN-tip 20 was equilibrated by applying 1 ml of Buffer QBT and letting the column to empty by gravity-flow. Then, the supernatant from the above step was applied to the QIAGEN-tip 20 and was allowed to enter the resin by gravity-flow. One milliliter of Buffer QC was used to wash the QIAGEN-tip 20 for 4 times and DNA was then eluted with 0.8 ml of Buffer QF. After that, DNA was precipitated with 7 volumes of room temperature isopropanol and immediately centrifuged at 10,000 rpm for 30 minutes in a microcentrifuge, and the supernatant was then discarded. DNA was washed with 1 ml of 70% ethanol, and air-dried for 5 minutes. DNA was then re-dissolved in a suitable volume of buffer. The yield of DNA was determined by quantitative analysis on an agarose gel. The purified product was performed with direct sequencing as explained above.

CHAPTER V
RESULTS

1. Analysis of *FLT3* internal tandem duplication (ITD) mutation by PCR and direct sequencing.

Agarose gel electrophoresis of PCR products of exon 14 to exon 15 (upper panel, Figure 12) and RT-PCR products of exon 12 to exon 16 of *FLT3* gene (lower panel, Figure 12) were shown. Sizes of PCR products with wild-type *FLT3* were 329 bp by PCR and 366 bp by RT-PCR. The *FLT3*-ITD amplification yielded a higher molecular weight product on a 2% agarose gel stained with ethidium bromide (Figure 12, Lane 6 and 8). There was no discrepancy between PCR and RT-PCR methods. 63 cases (24.6%) of the 256 cases had *FLT3*-ITD as shown in Figure 13. The majority of ITD cases contained more than one duplicated fragments although heteroduplex formation between wild-type and ITD fragments could be demonstrated in some cases by further sequencing analysis of individual bands. Two samples (lanes 4 and 27, Figure 13) displayed a low intensity of wild-type products while the remaining cases displayed equal or higher intensity of wild-type bands as compared to ITD bands. Peripheral blood mononuclear cells from 30 healthy donors were also studied using similar methods which revealed no ITD mutations.

The sequencing results from 14 representative ITD cases showed that the length of duplicated fragments varied from 21 to 201 base pairs (7 to 67 amino acids) and were all in-frame duplication (Figures 14, 15 and 16). The starting and ending sites of the duplications were different in each case, therefore, no identical duplication was observed. However, the target regions for duplication seemed to be restricted (Figure 15). In 13 out of 15 ITD fragments, the target sequences of duplication were restricted within exon 14. However, two ITD fragments (No. 2 and 3, Figure 15) included 3' part of exon 14, a whole intron and a 5' part of exon 15. The alignment of wild-type *FLT3* sequence as well as 15 other different types of *FLT3*-ITDmutations was shown

in Figure 16. The results showed that eight ITD products had extra amino acid of varying sizes inserted into the starting part of duplicated sequence. All ITDs (except fragment No. 4 included the duplication of at least one SH2 binding motif (YEYV and/or YEYDLK).

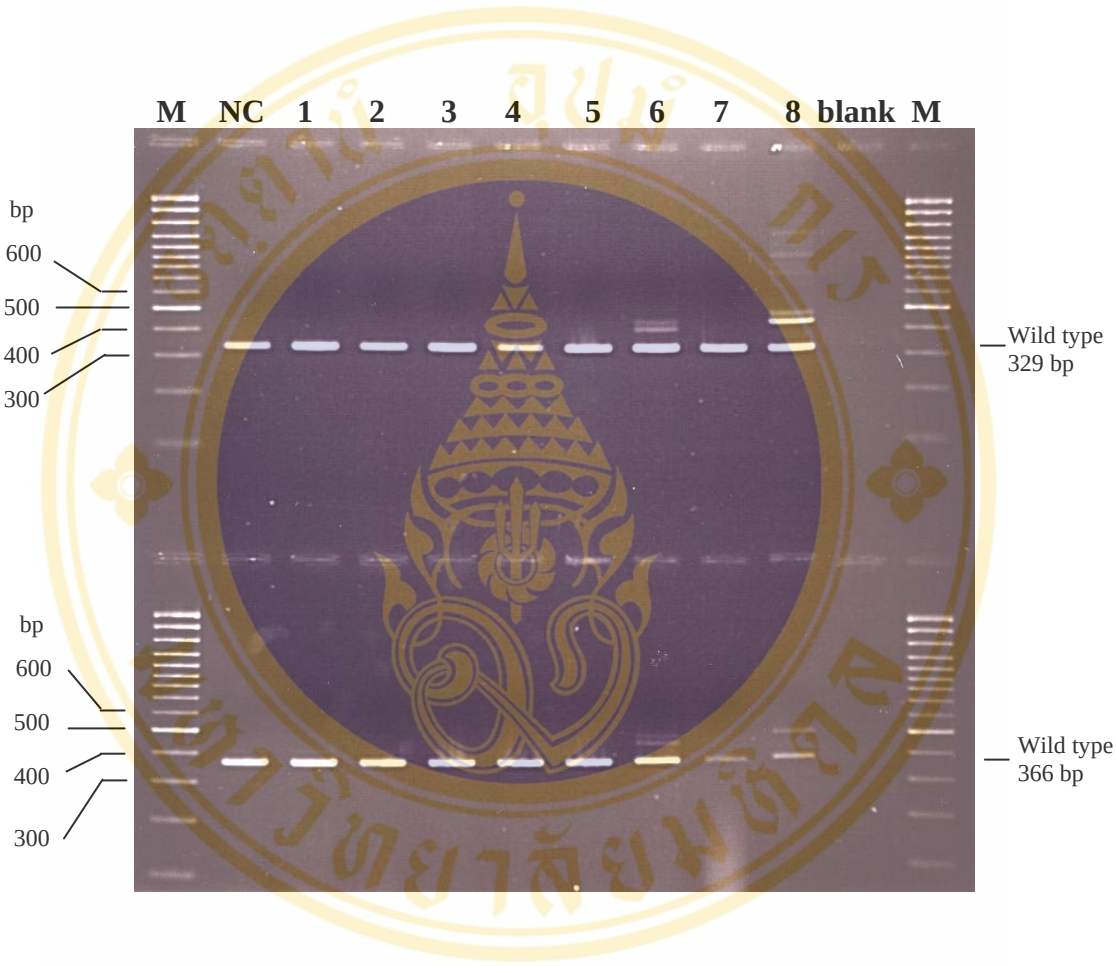

Figure 12 Agarose gel electrophoresis of PCR products (upper panel) of exon 14 to exon 15 and RT-PCR products (lower panel) of exon 12 to exon 16 of *FLT3* gene. Electrophoresis was performed on 2% agarose gel in 0.5X TBE buffer. Sizes of PCR products with wild-type *FLT3* were 329 bp by PCR and 366 bp by RT-PCR. The aberrant ITD products identified as the higer molecular weight band than the wild type (lane 6 and 8). Lane M is a 100-bp DNA ladder used as a standard-size marker. Lane NC and blank represent normal control and blank control, respectively. PCR products and markers were visualized by ethidium bromide staining.

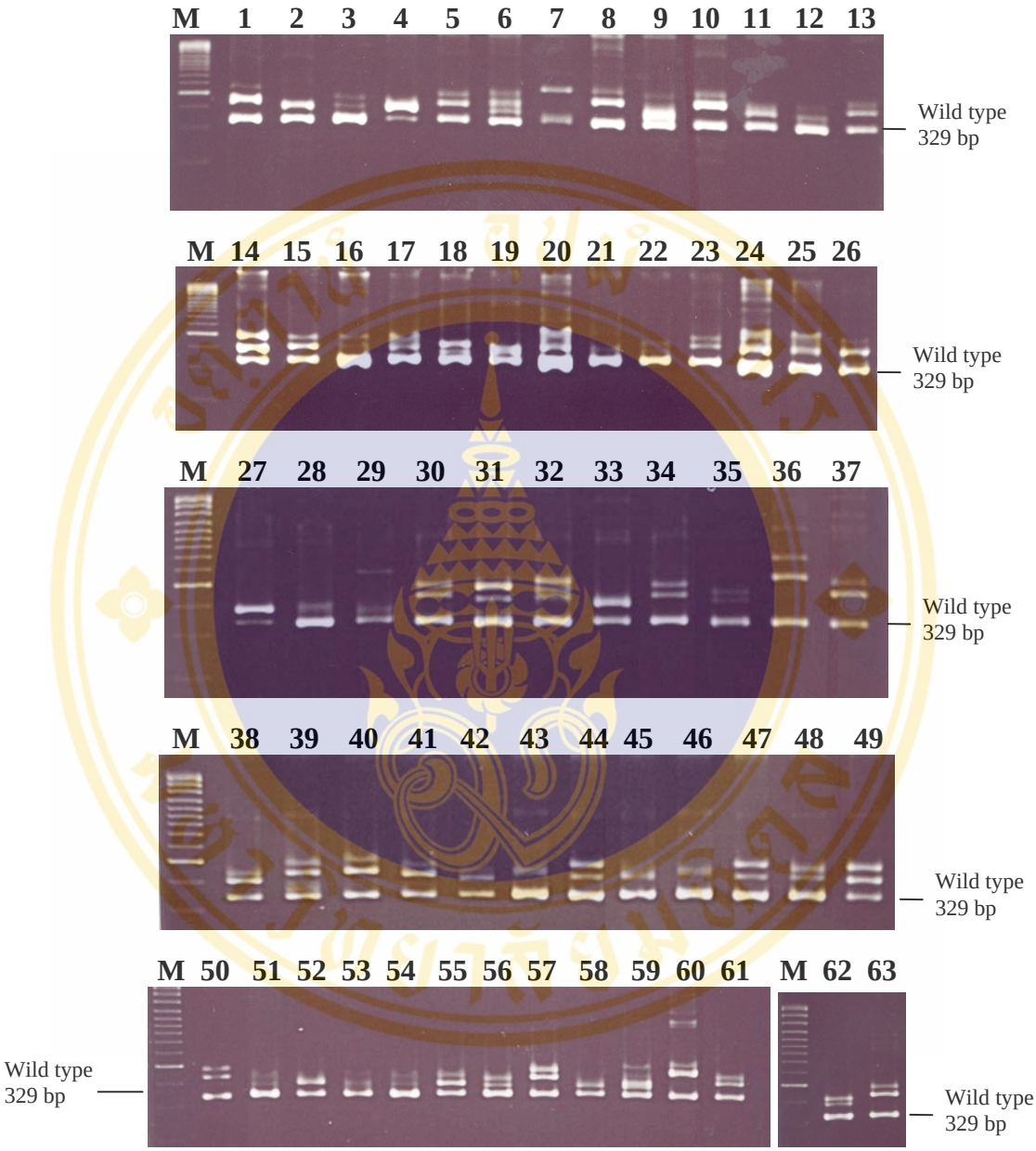

Figure 13 Agarose gel electrophoresis of PCR products from 63 cases with *FLT3*-ITD mutation. Electrophoresis was performed on 2% agarose gel in 0.5X TBE buffer. Sizes of PCR products with wild-type *FLT3* were 329 bp and the aberrant ITD products identified as the higher molecular weight bands than 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.

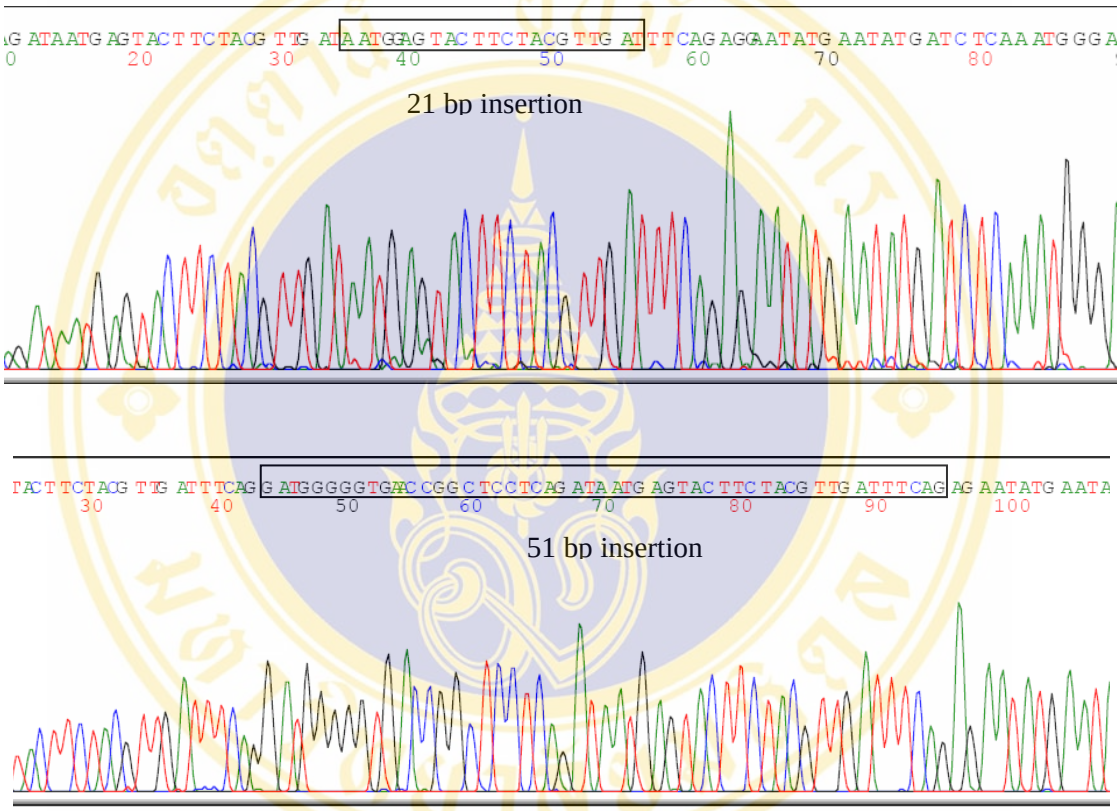

Figure 14 Sequencing profile from two representative cases containing *FLT3*–ITD mutation. The nucleotide sequences in the boxes represent duplication sequences which inserted into the wild-type *FLT3* sequence.

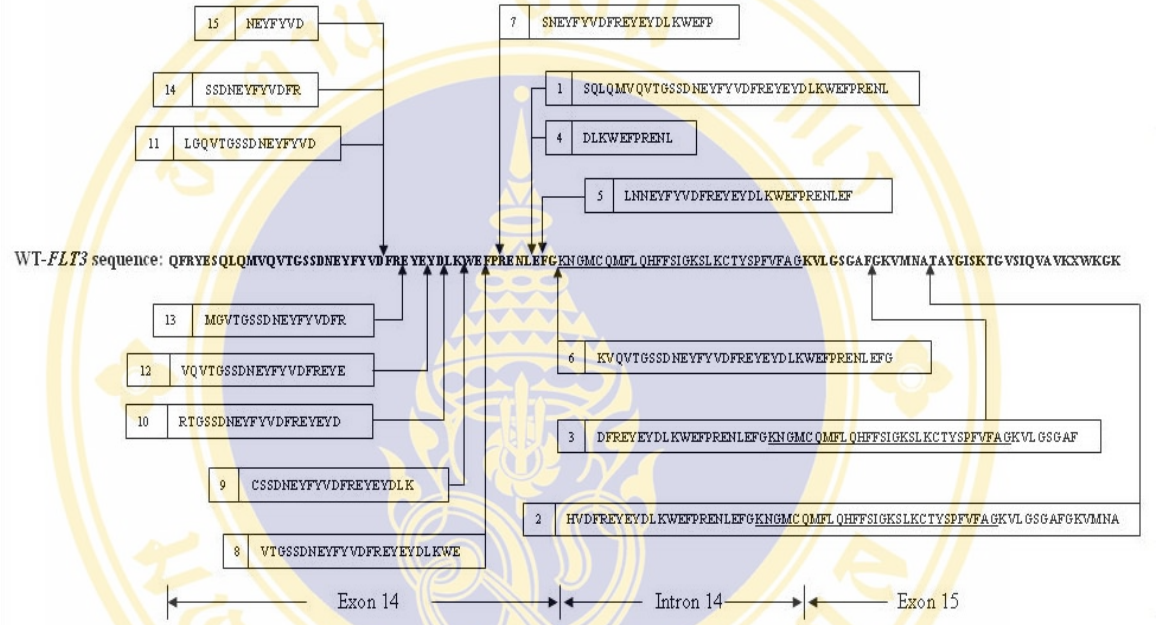

Figure 15 Internal tandem amino acid duplication within a part of exon 14 through exon 15 of *FLT*3 gene from 14 representative ITD cases (Fragments No. 1-15). The bold texts represent a whole part of exon 14, intron 14 and 5' part of exon 15 of wild-type *FLT3* sequence. The amino acid sequences in the boxes represent duplicated sequences of each of ITD fragment. The position of insertion regions were indicated by arrow.

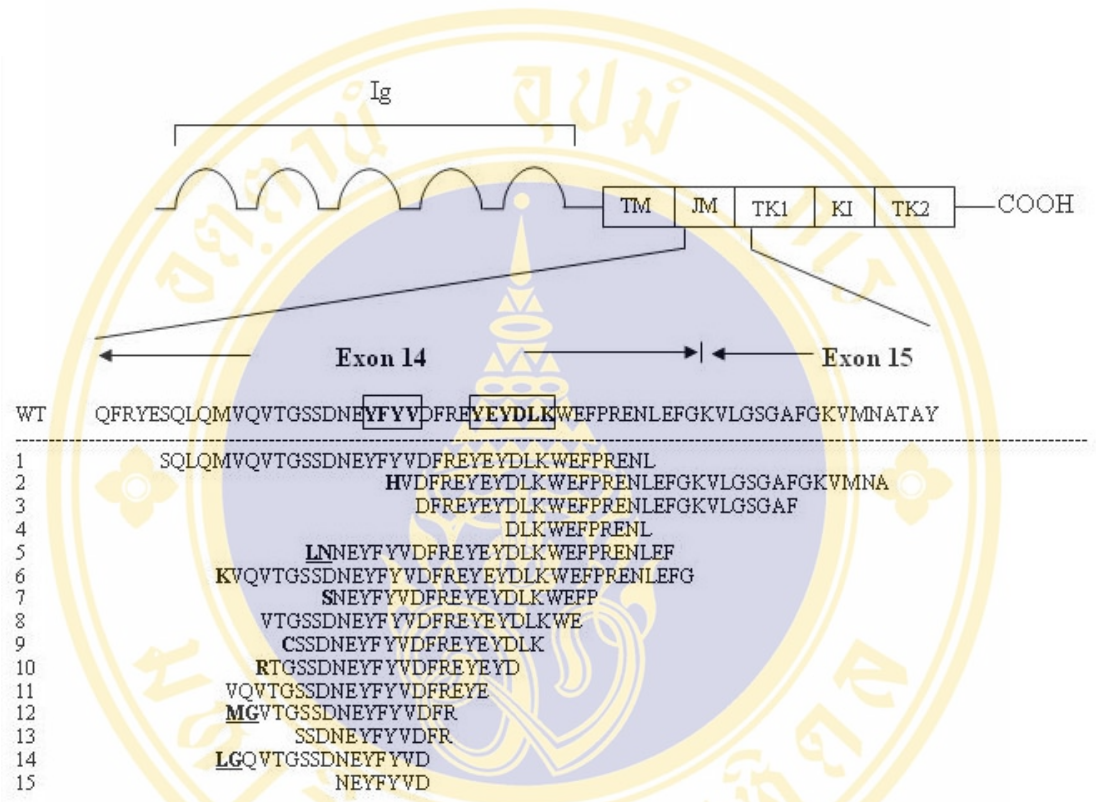

Figure 16 The length of duplication within JM and 5' TK1 domains is matched with the wild-type *FLT3* sequence. The bold and underlined bold texts represent amino acids which were substituted or added by insertion of nucleotides. The letters in the boxes indicate the minimum amino acid sequences which were duplicated frequently and also the binding domain for SH2 containing intracellular signaling proteins. Abbreviation: immunoglobulin-like domains (Ig), transmembrane (TM), tyrosine kinase (TK) and kinase insert (KI) domain.

2. Analysis of *FLT3* tyrosine kinase domain (TKD) mutation by PCR-RFLP and direct sequencing.

To analyse the alteration in exon 20 of *FLT3* gene, a 114-bp fragment comprised of 104 bp of the 3' end of exon 20 and 10 bp of 5' end of intron 20 was amplified by PCR (Figure 17, upper panel) and then digested with the EcoRV endonuclease. 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 17, lower panel). Fifteen of 256 (5.9%) AML cases had TKD mutation. However, 7 out of all TKD mutants included cases with ITD mutation (dual mutation). Therefore, 8 of AML cases (3.1%) contained isolated TKD mutation.

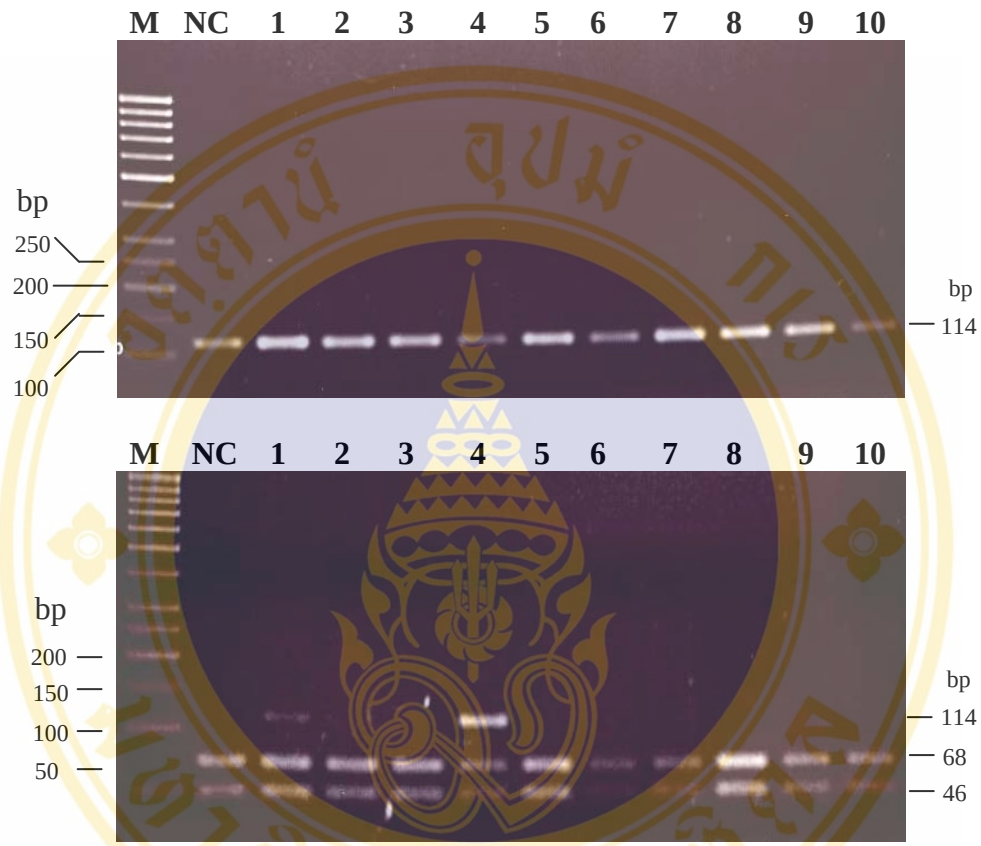

Figure 17 Agarose gel electrophoresis of PCR (upper panel) and PCR-RFLP (lower panel) products of exon 20 of *FLT3* gene. The PCR product size was 114 bp. The amplified products of the wild-type were digested into 2 bands (68 bp and 46 bp) by *EcoR*V whereas the amplified products containing TKD mutation had undigested band (114 bp) (lane 1 and 4). Lane M is a 50-bp DNA ladder used as a standard-size marker. PCR products and markers were visualized by ethidium bromide staining.

Sequence analysis showed that there were several kinds of TKD mutations, including missense and deletion mutation (Table 4, Figures 18 and 19). The first nucleotide G of codon 835 was most frequently substituted with T (46.6% of total cases), resulting in amino acid aspartic acid (Asp) changed to tyrosine (Tyr) (Asp835Tyr or D835Y). The C substitution for the first nucleotide G of codon 835 was the second most common type in 4 AML patients that resulted in Asp changed to histidine (His) (Asp835His or D835H). Furthermore, Asp835Glu (D835E) and Asp835Ala (D835A) TKD mutation was found in one patient each. One case of a previously described mutation, Ile836 deletion (I836del) was also found in this study. Interestingly, the novel mutation, Asp835 deletion + Ile836Val (D835del + I836V), had a deletion of ATA nucleotide, which occurred at a position between codon 835 and 836, resulting in loss of Asp and isoleucine (Ile) changing to valine (Val) amino acid, was identified in this study. The deletion mutation of this novel D835del + I836V was confirmed by cloning the undigested fragments into PCR 2.1 TOPO vector and subjected to sequencing (Figure 19, lower panel). Both deletion mutants (D835del + I836V and I836del) were found only in APL patients (Table 4).

Table 4 Type and frequency of *FLT3*-TKD mutations in 256 Thai adult AML patients

Mutation	Amino acid change	Total (256 cases)		Non-APL (216 cases)		APL (40 cases)	
		N	%	N	%	N	%
GAT → TAT	Asp835 → Tyr	7	46.6	6	50.0	1	33.3
GAT → CAT	Asp835 → His	4	26.6	4	33.3	0	0
GAT → GAG	Asp835 → Glu	1	6.7	1	8.35	0	0
GAT → GCT	Asp835 → Ala	1	6.7	1	8.35	0	0
I836 deletion	GAT ATC ATG → GAT --- ATG	1	6.7	0	0	1	33.3
D835del + I836V*	GAT ATC ATG → G-- -TC ATG	1	6.7	0	0	1	33.3

* Novel mutation

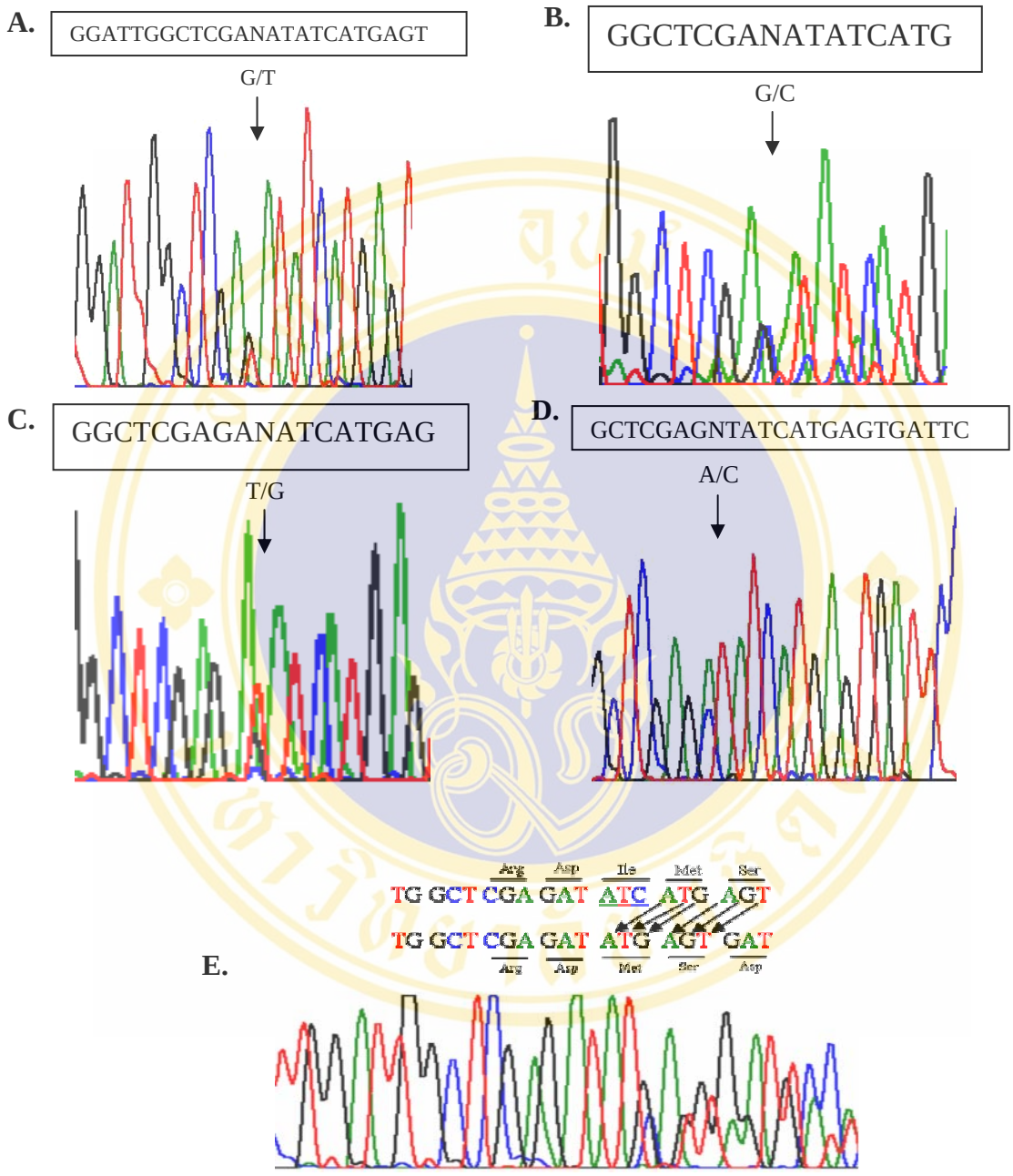

Figure 18 DNA sequencing profiles of the *FLT3* gene exon 20. The sequencing results showed a reported nucleotide substitution mutation at codon 835, resulting in the replacement of aspartic acid by (A.) tyrosine (G→T), (B.) histidine (G→C), (C.) glutamine (T→G) and (D.) alanine (A→C), respectively. In addition, (E.) *FLT3* I836 deletion was also identified in this study.

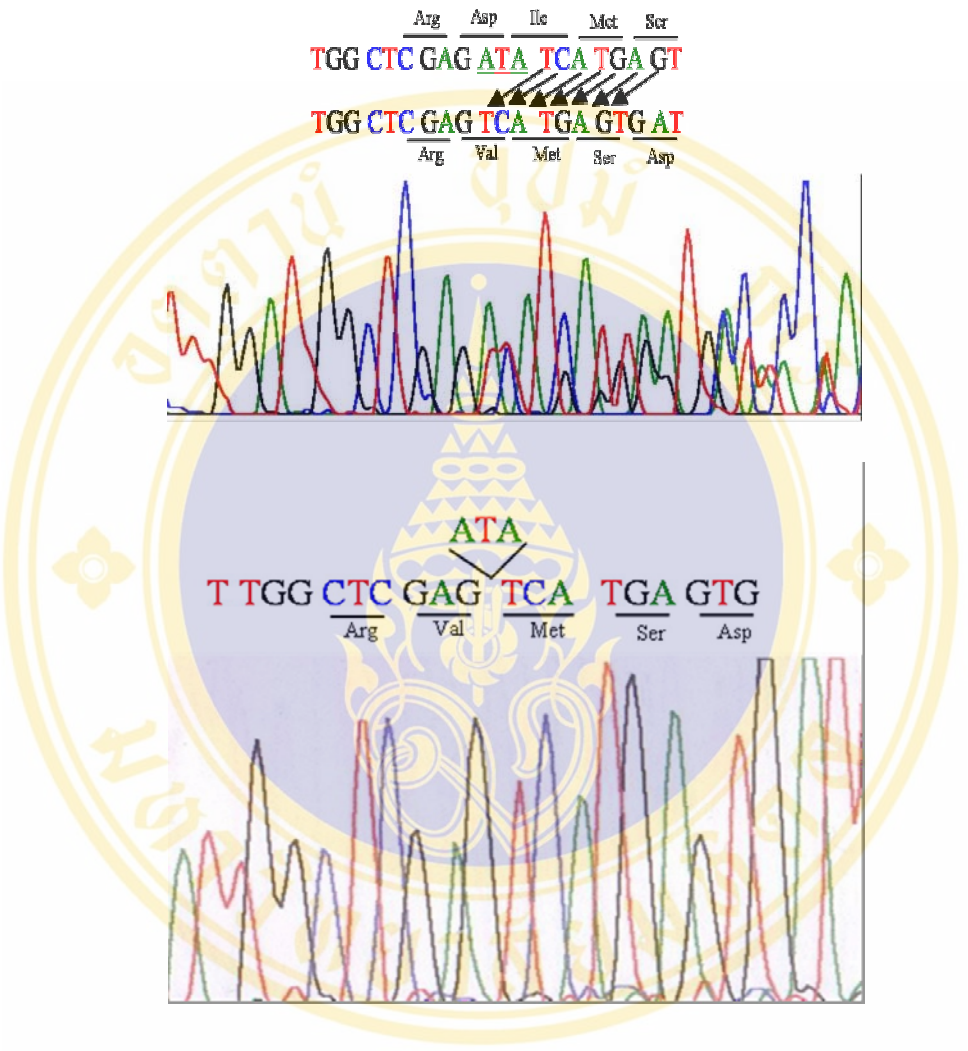

Figure 19 Sequencing profile of the novel *FLT3* D835del + I836V from amplified product (upper panel) and cloned plasmid DNA (lower panel) from a novel mutant patient.

3. Analysis of *FLT3* dual mutation by PCR-RFLP and direct sequencing.

Seven of 256 cases contained both ITD and TKD mutations. To determine whether both ITD and TKD mutation occurred on the same allele, the diagnostic RNA sample from one patient (lane 2 of Figure 20) who harbored both ITD and TKD mutations was available for analysis with the long template PCR assay using R5 and MR2 primers followed by *EcoR*V digestion and agarose gel electrophoresis. Figure 21 showed *FLT3*-ITD and *FLT3*-TKD occurred in different alleles. The amplified product with *FLT3*-ITD (upper band in lane 2; 1,263 bp) was completely digested by *EcoR*V as show by the middle band (1,091 bp) in lane 4. The lower band in lane 2 (1,191 bp) contained both wild type *FLT3* and *FLT3*-TKD products. Lane 4 showed the digested product of wild type *FLT3* and undigested *FLT3*-TKD, respectively.

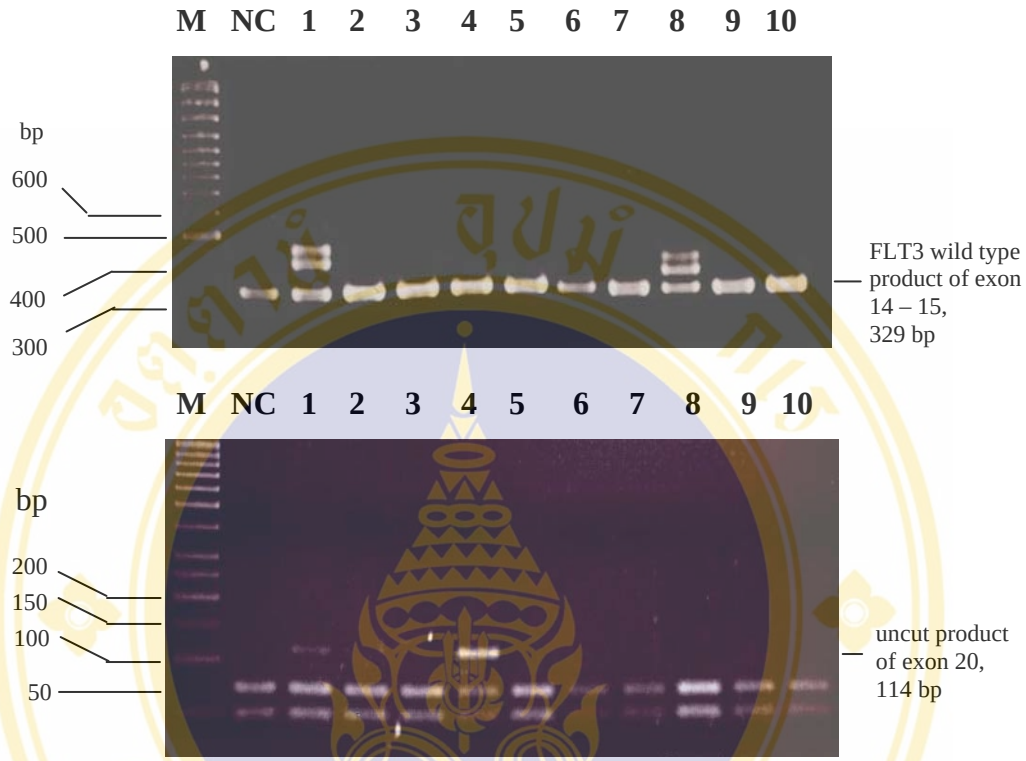

Figure 20 PCR analysis of *FLT3*-ITD and *FLT3*-TKD mutations. Upper panel showed agarose gel electrophoresis of PCR products with (lanes 1 and 8) and without *FLT3*-ITD mutation. The same set of samples was analyzed for *FLT3*-TKD mutation (lower panel). Lanes 1 and 4 showed undigested bands, arguing for a mutation. Sample in lane 1 showed both ITD and TKD mutation.

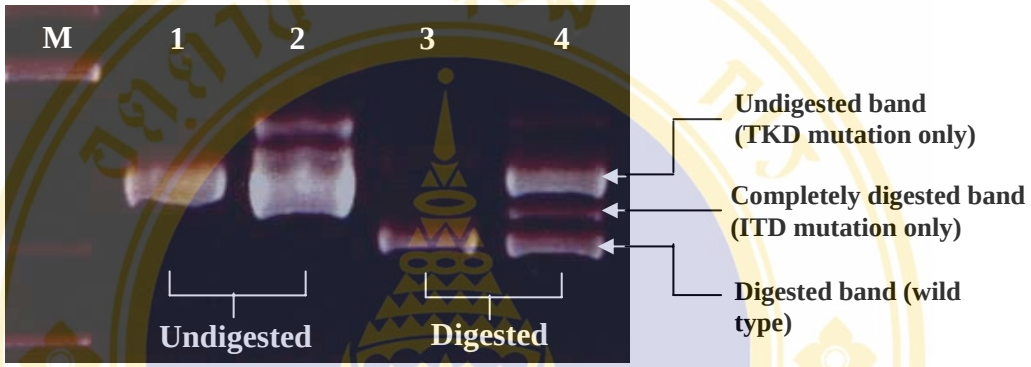

Figure 21 RFLP-mediated RT-PCR assay demonstrating that the ITD and TKD mutation occurred on a different allele in this particular patient; Lanes 1 and 3 represent the unmutated wild type products from a patient without *FLT3* mutation, while lanes 2 and 4 represent the patient with *FLT3*-dual mutants. *FLT3*- ITD (upper band, lane 2) was completely digested by *EcoR*V as show by the middle band in lane 4.

4. Incidence of *FLT3* mutations

A total of 256 samples were screened for the presence of *FLT3* mutations (Table 5). An ITD was found in 63 of total 256 AML cases (24.6%). A mutation in the TKD was present in 8 of these AML cases (3.1%). Seven AML patients had an *FLT3* ITD and *FLT3*-TKD in the same case. The *FLT3*-ITD was commonly found in APL patients (35% of APL cases). Thus, 78 patients (30.5%) of 256 AML cases were found to have such *FLT3* mutations.

Table 5 Frequency of *FLT3* mutations in 256 Thai adult AML patients.

Type of mutation	Total (256 cases)		Non-APL (216 cases)		APL (40 cases)	
	N	% [a]	N	% [a]	N	% [a]
ITD only	63	24.6	49	22.7	14	35.0
TKD only (D835 or I836 mutation)	8	3.1	6	2.8	2	5.0
Dual mutation (ITD with TKD mutation)	7	2.7	6	2.8	1	2.5

[a] Calculated as a percentage of a whole group

5. Clinical characterization of Thai AML patients with or without *FLT3* mutations

To determine if clinical differences between 216 non-APL patients with and without *FLT3* mutations exist, the patients' clinical characteristics at the initial diagnosis were compared (Table 6). The presence of any type of *FLT3* mutations was markedly associated with high leukocyte counts as compared to the wild-type *FLT3* cases. No association was seen between the mutant and wild type group in term of hemoglobin (Hb) and platelet (Plt) count. *FLT3* mutations were frequently found in the older patients with more than 30% of the patients being older than 60 years old. In contrast, the majority of the wild-type *FLT3* patients were younger than 60 years old (78.3%). Isolated *FLT3*-ITD and wild-type *FLT3* group had a slight female preponderance while the *FLT3*-TKD and dual mutation group predominantly had more males. In addition, the similarly result of APL also shown in Table 7. However, no statistical significance could be demonstrated in any parameter.

Of 256 AML cases, the majority of patients with mutant *FLT3* (48.7%) or wild-type *FLT3* (60%) were categorized as M1 or M2 subtype according to the French–American–British (FAB) Classification, with a slightly larger proportion of the mutant *FLT3* cases categorized as M4 or M5 subtype than the wild-type (29.5% vs 22.5%, respectively). Significantly more APL cases were also identified in the mutant *FLT3* (21.8%) as contrast to the wild-type *FLT3* (12.9%) (Table 8).

Table 6 Demographic data of 216 Thai AML patients with or without *FLT3* mutations.

Parameter	ITD only (49 cases)	TKD only (6 cases)	Dual (6 cases)	All mutants (61 cases)	Wild-type (155 cases)
Median age (year)	49	38	40	48	42
Age range (year)	15-76	13-66	16-74	13-76	13-85
Age>60 yrs	31.3	33.3	33.3	31.7	21.7
Male (%)	49.0	66.7	66.7	52.5	43.9
Median WBC ($x10^9$/L)	66.2 [a]	87.6	48.8	66.2 [a]	24.0 [a]
% of patients with WBC count <20 $x10^9$/L	18.4	33.3	16.7	19.7	46.0
% of patients with WBC count 20-50 $x10^9$/L	24.5	16.7	33.3	24.6	25.3
% of patients with WBC count >50 $x10^9$/L	57.1 [b]	50.0	50.0	55.7 [b]	28.7 [b]
Median Hb (g/dL)	7.2	6.7	7.3	7.1	7.6
Median Plt ($x10^9$/L)	51.0	44.7	60.0	51.0	45.0

[a] P< 0.001 ; Mann Whitney U test

[b] P< 0.001 ; Chi-square test compare frequency between different group

Table 7 Demographic data of 40 Thai APL patients with or without *FLT3* mutations.

Parameter	ITD only (14 cases)	TKD only (2 cases)	Dual (1 cases)	All mutants (17 cases)	Wild-type (23 cases)
Median age (year)	48	60	35	50	45
Age range	19-77	50-71	ND	19-77	15-84
Age>60 yrs	28.6	50.0	100	29.4	34.8
Male (%)	28.6	0	0	23.5	39.1
Median WBC ($x10^9$/L)	44.9	111.4	25.3	45.9	26.4
% of patients with WBC count <20 $x10^9$/L	28.6	0	0	23.5	43.5
% of patients with WBC count 20-50 $x10^9$/L	35.7	0	100	35.3	21.7
% of patients with WBC count >50 $x10^9$/L	35.7	100.0	0	41.2	34.8
Median Hb (g/dL)	9.4	6.3	ND	8.9	7.1
Median Plt ($x10^9$/L)	49.0	63.0	85.0	52.0	38.6

ND = data not available

Table 8 FAB subtypes of 256 AML patients with or without *FLT3* mutations**.**

FAB	ITD only (63 cases)		TKD only (8 cases)		Dual (7 cases)		All mutant (78 cases)		Wild type (178 cases)	
	N	%	N	%	N	%	N	%	N	%
M1/M2	31	49.2	4	50.0	3	42.9	38	48.7	107	60.1
M3	14	22.2	2	25.0	1	14.2	17	21.8	23	12.9
M4/M5	18	28.6	2	25.0	3	42.9	23	29.5	40	22.5
M6	0	0	0	0	0	0	0	0	7	3.9
M7	0	0	0	0	0	0	0	0	1	0.6

6. Cytogenetics and prognosis of Thai AML patients with or without *FLT3* mutations

Correlation of cytogenetic data in patients with and without *FLT3* mutations is shown in Table 9. Most *FLT3* mutations were observed in patients with a normal karyotype. The chromosome abnormalities that were commonly found in patients with isolated *FLT3*-ITD were trisomy 8 and t(15;17), respectively. The isolated *FLT3*-TKD was found in one case with trisomy 4 and t(8;21). Interestingly, the rare abnormal chromosome, trisomy 11, was also found in one case with a novel FLT3-D835del + I836V. t(8;21) was the only abnormal karyotype found in *FLT3*-dual mutation. In contrast to *FLT3* mutated cases, *FLT3* wild type cases had variable cytogenetic abnormalities. The majority of *FLT3* mutation cases were found to be in the standard risk group according to the MRC criteria. None of the mutated cases were found in the poor risk group.

Moreover, the presence of the different types of *FLT3* mutations was correlated to survival time, although, the patients with AML-M3 (APL) were analyzed separately because they had been treated according to different protocol. As shown in Figure 22, the presence of *FLT3* mutation was associated with shorter overall survival than the wild type group. However, these differences were statistically significant only in ITD patients. No significant difference was seen in patients with both type of mutations (TKD and dual), however this may be a result of the small sample size. In addition, the similarly result of APL cases also shown in Figure 23. Interestingly, the result from non APL with standard risk group showed the significantly different survival between samples with or without ITD mutations (Figure 24).

Table 9 Incidence of chromosome abnormalities in 256 Thai Adult AML patients with or without *FLT3* mutations**.**

Karyotype	ITD only (63 cases)		TKD only (8 cases)		Dual Mutations (7 cases)		Wild-type (178 cases)	
	N	%	N	%	N	%	N	%
normal karyptype	44	69.8	4	50.0	4	57.1	81	45.5
t(8;21)	1	1.6	1	12.5	2	28.6	19	10.7
t(15;17)	2	3.2	0	0	0	0	6	3.4
inv(16)	0	0	0	0	0	0	5	2.8
t(9;22)	0	0	0	0	0	0	4	2.2
-5/-7	0	0	0	0	0	0	11	6.2
del(11)(q23qter)	0	0	0	0	0	0	3	1.7
del(9)	1	1.6	0	0	0	0	3	1.7
+8	3	4.8	0	0	0	0	7	3.9
+11	0	0	1	12.5	0	0	2	1.1
+21	0	0	0	0	0	0	2	1.1
+22	0	0	0	0	0	0	2	1.1
+4	0	0	1	12.5	0	0	1	0.6
other abnormalities	2	3.2	0	0	0	0	20	11.2
Chromosome analysis not performed	9	14.3	1	12.5	1	14.3	9	5.1
Data not available	1	1.6	0	0	0	0	3	1.7

Table 10 Risk groups of Non-APL patients with or without *FLT3* mutations**.**

Risk group according to MRC criteria	ITD only (49 cases)		TKD only (6 cases)		Dual mutations (6 cases)		All mutants (61 cases)		Wild-type (155 cases)	
	N	%	N	%	N	%	N	%	N	%
Good	1	2.4	1	20	2	40	4	7.7	24	16.6
Standard	41	97.6	4	80	3	60	48	92.3	106	73.1
Poor	0	0	0	0	0	0	0	0	15	10.3
Insufficient metaphase	7	14.3	1	16.7	1	16.7	9	14.8	10	6.5

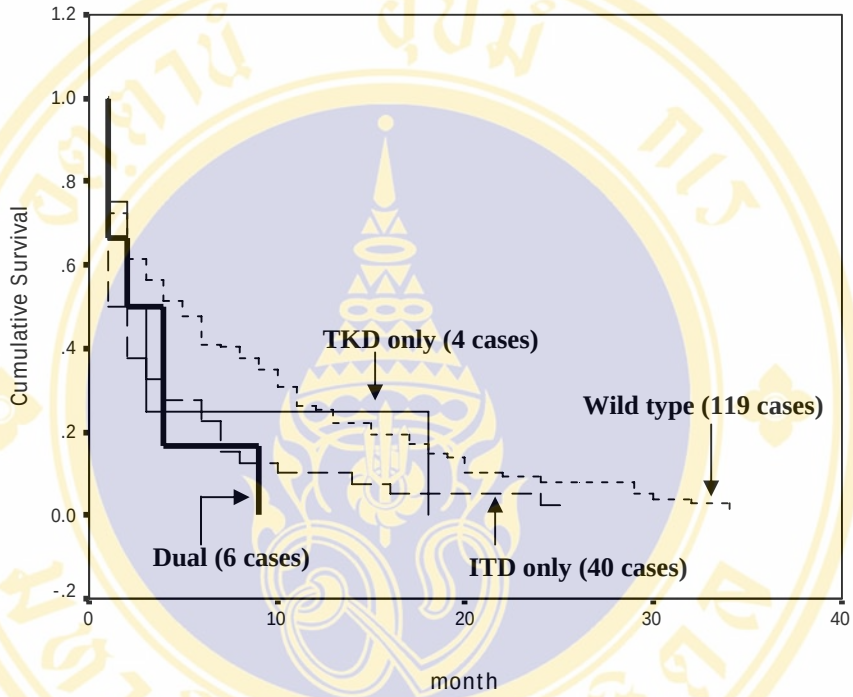

Figure 22 Kaplan-Meier cumulative survival curves of Thai AML patients (APL cases excluded) with *FLT3*-ITD, *FLT3*-TKD, dual mutant or *FLT3*- wild-type. Median overall survival was 4.35, 6.0, 3.50 and 8.51 months, respectively (statistically significant only between isolated ITD and wild-type cases; p value = 0.0039)

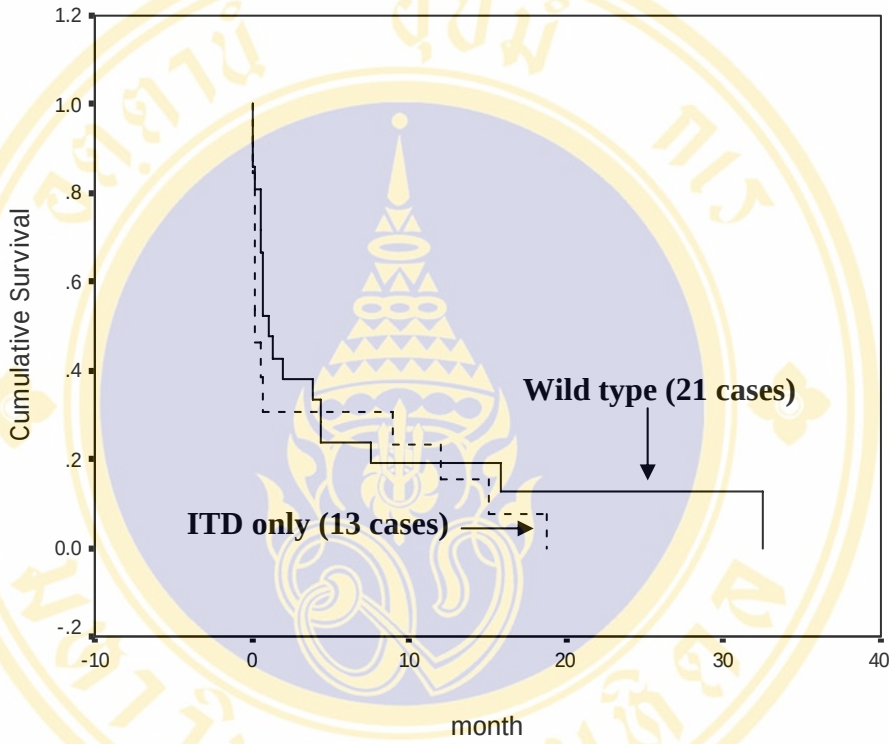

Figure 23 Kaplan-Meier cumulative survival curves of Thai APL patients with FLT3-ITD or FLT3 wild-type. Median overall survival was 4.38 and 6.50 months, respectively (p value = 0.33)

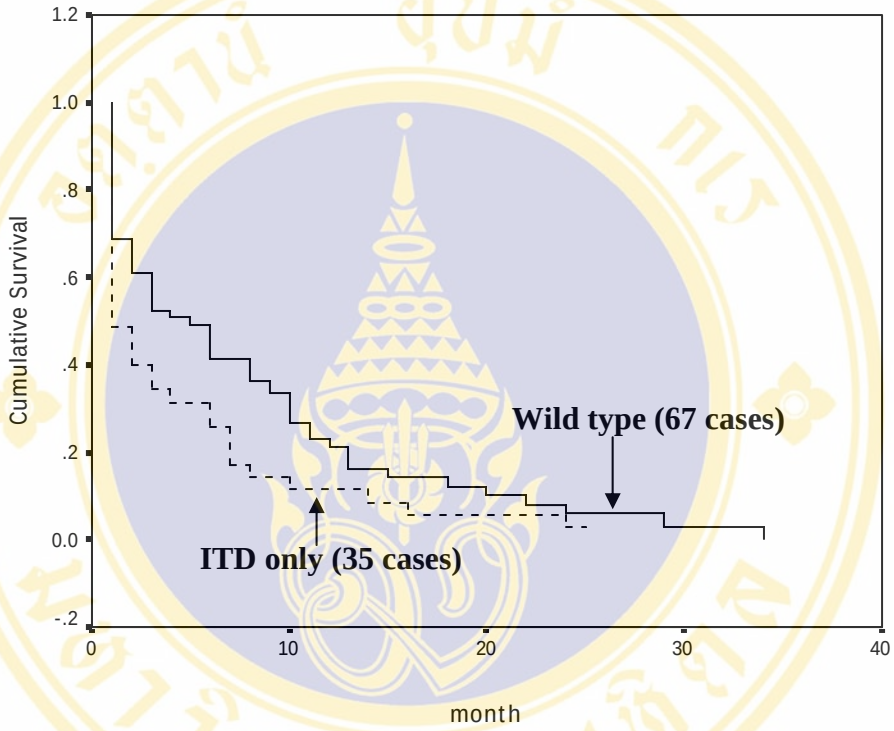

Figure 24 Kaplan-Meier cumulative survival curves of intermediate risk group Thai AML patients (APL cases excluded) with FLT3-ITD or FLT3 wild-type. Median overall survival are 4.69 and 7.68 months, respectively (p value = 0.05)

7. Immunophenotypic characteristics of *FLT3* mutations

The immunophenotypes of 256 AML patients with or without *FLT3* mutations (ITD, TKD and dual mutants) demonstrated high expression of myelomonocytic antigens, including CD33, CD13, CD11b, CD64 and anti-myeloperoxidase (anti-MPO). Additional antigens that were frequently expressed were CD11c and HLA-DR. In contrast to the consistent myelomonocytic pattern, expression of early stem cell and lymphoid antigen were variable. CD4, CD8, CD10 and CD20 were always negative in all mutant cases (Table 11).

The antigenic expression of each *FLT3* mutations group was compared with that of the wild-type *FLT3*. The isolated *FLT3*-ITD cases weakly expressed CD15 and CD34, but statistical significance was only found in CD15 ($p<0.05$). Moreover, CD15 was negatively expressed in both TKD and dual mutation. The *FLT3* dual mutation cases frequently expressed B-cell antigen (CD19) ($p<0.001$) than any *FLT3* mutant cases and also greater expressed CD19 than both ITD and TKD mutant.

Table 11 Immunophenotypes of 256 Thai AML patients with or without *FLT3* mutations.

Antigen	ITD only (63 cases)			TKD only (8 cases)			Dual mutations (7 cases)			Wild-type (178 cases)		
	Pos./Ex.	%pos.	Mean express	Pos./Ex.	%pos.	Mean express	Pos./Ex.	%pos.	Mean express	Pos./Ex.	%pos.	Mean express
CD34	36/63	57.1	39.38	7/8	87.5	60.46	5/7	71.4	47.72	115/177	65.0	92.53
CD33	60/63	92.3	86.33	8/8	100.0	85.01	7/7	100.0	94.06	162/177	91.5	75.71
CD13	56/60	93.3	70.76	8/8	100.0	72.23	6/6	100.0	86.83	167/175	95.4	75.76
CD14	12/62	19.3	12.52	3/8	37.5	16.05	3/7	42.8	29.90	46/177	26.0	14.76
CD11b	27/41	65.9	36.77	5/7	71.4	32.78	5/6	83.3	51.17	76/138	55.1	35.66
CD11c	26/30	86.7	55.74	3/3	100.0	81.36	2/2	100.0	54.75	81/97	83.5	50.56
HLA-DR	46/63	73.0	61.94	6/8	75.0	47.64	6/7	85.7	72.93	148/177	83.6	61.66
CD64	6/8	75.0	53.22	1/1	100.0	94.61	0/1	0	0.23	5/12	41.7	33.58
CD117	6/8	75.0	45.23	1/1	100.0	54.18	1/1	100.0	95.17	8/12	66.7	48.51
GPA	0/59	0	2.54	0/8	0	3.11	0/7	0	3.04	14/175	8.0	6.93
CD41a	3/62	4.8	4.85	0/8	0	3.88	2/7	28.6	11.53	7/175	4.0	4.61
MPO	22/26	84.6	34.91	2/3	66.7	12.73	1/2	50.0	6.52	65/80	81.3	41.23
TdT	2/26	7.7	6.68	0/3	0	0.50	1/2	50.0	16.70	4/80	5.0	4.43
CD2	1/56	1.8	2.43	0/7	0	1.80	0/7	0	1.40	3/169	1.8	2.70
CD3	2/63	3.2	3.49	1/8	12.5	11.90	0/7	0	2.11	4/177	2.3	2.90
CD4	0/30	0	1.03	0/5	0	0.50	0/5	0	0.86	1/84	1.2	1.66
CD5	1/63	1.6	1.83	0/8	0	2.42	1/7	14.3	7.81	3/177	1.7	2.76
CD7	5/63	7.9	6.24	0/8	0	1.55	0/7	0	5.40	27/177	15.3	9.96
CD8	0/30	0	1.33	0/5	0	2.39	1/5	20.0	6.57	0/84	0	2.16
CD10	0/63	0	0.42	0/8	0	0.36	0/7	0	0.21	2/176	1.1	1.07
CD15	1/46	2.2	3.68	0/3	0	4.31	0/6	0	4.83	21/152	15.8	12.31
CD19	23/63	36.5	25.19	2/8	25.0	19.65	6/7	85.7	57.05	47/176	26.7	21.10
CD20	0/63	0	1.03	0/8	0	1.29	0/7	0	1.25	0/177	0	1.14
CD22	8/63	12.7	9.04	2/8	25.0	17.98	3/7	42.9	16.98	16/175	9.1	6.50
CD16	6/54	11.1	9.85	1/6	16.7	15.52	2/7	28.6	13.13	23/160	14.4	10.07
CD56	23/59	39.0	28.96	2/8	25.0	17.93	3/7	42.9	29.38	64/167	38.3	27.20
CD38	29/32	90.6	65.61	5/5	100.0	62.76	5/5	100.0	74.88	63/84	75.0	60.38

CHAPTER VI
DISCUSSION

In this study, I evaluated the frequency and type of *FLT3* mutations in 256 newly diagnosed Thai adult AML patients. The most common *FLT3* gene alteration in Thailand was the mutation in the JM domain with a less frequent mutation in the second tyrosine kinase domain. *FLT3*-ITDs were more frequently found in APL cases when compared to non-APL cases, which were in agreement with other previous reports (2, 14, 15, 121, 151). Thai mutant *FLT3*-ITD patients were slightly older than the WT patients (median age of 49 vs 42, respectively), although both groups appeared to be much younger than AML patients in the western countries.

The incidence of *FLT3* mutations in Asian countries was previously known only for patients in Japan and Taiwan. Half of the published Japanese studies were adult AML series. The frequency of *FLT3*-ITD in adult AML patients from these Japanese studies was between 14 to 23% (10, 125, 152). Two studies from Taiwan reported the incidence of both ITD (15.4 and 20.6%) and D835 mutations (3.3 and 18.7%), of which one study reported only pediatric AML cases (n=91) and the other reported only APL cases (n=107) (153, 154). The frequency of *FLT3*-ITD in AML patients in Thailand was thus similar to that of Japan, Taiwan and other countries from Europe and North America, despite the geographic and ethnic differences. At present, no data is currently available in other Asian countries for full comparison. Our study represents the largest and first series of *FLT3* mutations ever reported from Thailand and the Southeast Asian region.

Table 12 Incidence of *FLT3* mutations in adult and pediatric AML patients from various countries.

Country	No. of AML patients	Incidence of *FLT3*-ITD (%)	Incidence of *FLT3*-TKD mutations (%)
Asia			
Japan			
Nakao et al (1996) (125)	30 (adult)	23	N/A
Yokota et al (1997) (152)	112 (adult)	20	N/A
Kiyoi et al (1997) (155)	74 (APL only)	20.3	N/A
Iwai et al (1999) (156)	94 (pediatric)	5.3	N/A
Xu et al (1999) (157)	87 (pediatric)	13.8	N/A
Kondo et al (1999) (158)	64 (pediatric)	11	N/A
Matsuno et al (2003)(150)	49 (adult)	14	8
Matsuno et al (2005)(149)	172 (adult)	N/A	9.3
Taiwan			
Liang et al (2003) (154)	91 (pediatric)	15.4	3.3
Shih et al (2003) (153)	107 (APL only)	20.6	18.7
Korea			
Kang et al (2005) (159)	61 (pediatric non APL)	6.6	3.3
Thailand			
This study (2005)	256 (adult)	27.3	5.9
Europe and North America *(2000-2005)* (2, 14, 48, 151, 160-162)	34-1,003	10-25.1%	5-7.7%

The structural alterations of the *FLT3* gene were characterized in our study. In the present study, ITDs always led to intact in-frame transcription and the mutant ITD sequences were unique in each case with the insertion sequences varied from 21 to 201 bp and 7 to 67 amino acids. In Germany, the insertion sequences varied from 6 to 180 bp in one study and from 3 bp to more than 400 bp in another study (2, 15). Similar results were also reported from the UK and Italian study (2, 14). In Japan, the number of nucleotides added to the *FLT3*-wild type varied from 18 to 204 bp in one study and from 18 to 90 bp in another study (10, 152). The Taiwanese group also showed the duplicated sequence ranging in size from 18 to 105 bp (153). All of the ITD sequences, except case no. 4, contained about two to four Tyr residues. Interestingly, the SH2-binding motifs (YFYV and YEYDLK) were frequently involved in the ITD in our study (Figure 16). This motif is a putative binding site for other signaling

protein, suggesting that the duplication of the SH2-binding motifs in the JM domain might be associated with enhancement of the *FLT3* RTK function. The numbers of tyrosine residues involved in the duplication were also reported to correlate with the peripheral WBC counts. However, recent study demonstrated that not only the ITD, but also an elongating or shortening JM domain could be shown to transform murine IL3-dependent myeloid progenitor cell lines regardless of the tyrosine residues in the JM domain (131). The length-mutated JM domain not only induces ligand-independent receptor activation but also activates Wild type FLT3 in a trans-manner. Moreover, the insert domain of ITD may also disturb "*FLT3* autoinhibition" or by making *FLT3* autoinhibition "leaky", and thus allows *FLT3* to switch from the inactive to the active conformation without the presence of its endogenous ligand. Recent study of the crystal structure of *FLT3* supported this above hypothesis (138).

Several studies have indicated that patients with high ITD to wild-type *FLT3* ratios had the worst clinical prognosis. This study showed that two cases (case no. 4 and 27, Figure 13) displayed high ITD to wild-type ratio than other samples. The clinical impact of the ITD to wild-type ratio in this study is unknown given the limited number of cases. Whitman et al found that three patients with an extremely high ITD to wild-type *FLT3* ratios had loss of heterozygosity (LOH) of the wild-type *FLT3* allele, which indicates that LOH might explain some of the difference in the ITD to wild-type ratios among patients. Increased ITD to wild-type ratios might be due to homozygous recombination of the *FLT3*-ITD alleles, thereby creating more copies of the *FLT*3-ITD alleles than of wild-type allele. The most probable explanation for the variation of allelic ratio between leukemia cases lies in the observation that, in many cases, leukemia might be a polyclonal process. Some sub-populations of AML cells might have an *FLT3*-ITD, whereas others contain only wild-type *FLT3*. For example, *FLT3* mutational status can change between diagnosis and relapse, with some patients developing a new or different *FLT3* mutation on relapse, whereas other patients lose their *FLT3* mutation altogether. If leukemia contained two clones with different *FLT3* mutation status (one with cells containing an *FLT3* ITD, and one with wild-type *FLT3*), then patients having a higher percentage of clones with *FLT3*-ITDs would have higher ITD to wild-type ratios. If the cells with the *FLT3* ITD are more resistant

to therapy, then patients with higher ITD to wild-type ratios would be more likely to have a poor clinical outcome than patients with a lower ratio.

In our study, four known types of D835 mutations were found. Moreover, Ile836 deletion mutation was also identified. Interestingly we identified a *novel* deletion in codon 835 (D835del+I836V) which resulted in the loss of Asp and change of Ile to Val amino acid. This patient was a 71-year-old Thai woman who was also found to have trisomy 11 on her karyotypic analysis. Her WBC count was markedly elevated ($136.9x10^9$/L) with Hb of 7.56 g/dL and a platelet count of $78x10^9$/L and her overall survival was less than 1 month. This novel D835 mutation was thus associated with markedly elevated WBC counts and reflects the typical leukocytosis pattern frequently associated with other types of TKD mutations, although the prognostic significance of TKD mutations is presently unclear. To exclude the possibility of polymorphism, I also analyzed blood samples from normal individuals and found that TKD mutation was not found in any normal individual studied. Of interest is our finding that in this patient together with six additional patients harboring dual mutation, the ITD and TKD mutations were proven to occur independently on a different allele. This finding is consistent with all previously reported cases in the literature and argues that each *FLT3* variant may contribute to the molecular pathogenesis of AML. However, the other possibility regarding the defect of dual mutation could not be excluded. The dual mutation which displayed both ITD and TKD may have come from a sample contained two different clones of ITD and TKD. This study cannot definitely prove that both mutations came from a different allele of a single clone.

A number of studies have demonstrated that both adult and pediatric AML patients with activating mutations, particularly ITDs, have a worse prognosis than those without mutations. Several studies noted that ITDs were more frequently associated with the old age at diagnosis. German AML patients with mutant *FLT3* had the median age of 54.9 years old as contrast to 57.6 years old in the *FLT3*-wild type patients. Similar result was also reported from the American group. Our study showed that Thai mutant *FLT3* patients were slightly older than the non-mutant patients (mean age of 47 vs 43.7, respectively), although both groups appeared to be much younger than AML patients in Japan or other western countries. In the UK study, the OS in the

FLT3-ITD patients was only 4.6 months for mutant *FLT3* and 19.8 months for *FLT3*-WT. In this study, we revealed that our patients with *FLT3*-ITD also had worse overall survival than *FLT3*-WT, which is similar to other reports, although the overall survival of Thai AML patients as a whole group appeared to be much shorter than that of other developed countries despite the younger age at diagnosis.

In contrast to all AML patients, *FLT3*-ITD has been shown not to correlate with prognosis in the AML M3 subgroup (155). Because of differentiation therapy with all-trans retinoic acid, the prognosis for APL is favorable compared with other type of AML. Therefore the APL group has to be analyzed separately. In this study of 40 patients with M3 showed a trend toward inferior outcome in *FLT3*-ITD compare to wild type *FLT3* cases. However, it could not demonstrate a significant implication of them. The other type of *FLT3* mutations is too rare in APL thus it was not reasonable to compare the prognosis. As suggested in many reports, the high frequency of *FLT3* mutations, especially ITD, in patients without detectable cytogenetic aberrations of aberration of currently unknown prognostic significant may provided the mean to categorize this type of mutation into poor prognostic subgroup. This study showed the supported result, the normal karyotype patients with *FLT3*-ITD is associated with adverse outcome as displayed by shorter overall survival than wild type *FLT3* cases.

With respect to karyotypic association, most of our patients with *FLT3* mutations had a normal karyotype. Of the abnormal karyotypes found to coexist with *FLT3* mutations, t(15;17) was the most frequent. In a large German study, t(15;17) was found in 7.1 %, followed by t(6;9)(4.9%) and t(8;21)(1.1%) (15). In another study, the most frequent chromosome aberrations found together with *FLT3*-ITD were t(15;17) (10.2%), t(8;21) (2.6%), 11q23 (0.4%) and -5/-7 (0.4%) (163). One Spanish study reported that 3.4% of their AML cases had *FLT3* mutations and additional chromosome abnormalities, including inv(16) and t(9;11) (164). In our study, we found 6 cases of t(8;21), 3 cases of trisomy 8, 1 case of del(9) and 1 case of trisomy 11. Our findings thus provide preliminary evidence to support the existence of cooperativeness between mutations in the transcription factors such as t(8;21) and t(15;17) and signal transducer genes such as *FLT3* in a subset of Thai adult AML patients. This fits well with the molecular leukemogenesis model recently proposed by Gilliland et al (6, 47). Moreover, the coexistence of trisomy 11 with our novel

FLT3 mutation may suggest the potential association of the MLL gene on chromosome 11 with our novel *FLT3* mutation (165). Further comprehensive genotypic analysis of AML cells is needed to give more insights into the pathogenesis of AML in Thai patients. Of particular interest is the detection of patients in the standard risk karyotypic category (especially normal karyotype) that harbored ITD mutation. The presence of *FLT3*-ITD might allow the identification of a subset of poor prognosis among this standard or intermediate risk group. This finding is consistent with two British studies (166, 167). Abu-Duhier *et al* reported that *FLT3*-ITD was an important adverse prognostic factor among standard risk group as shown by significant shorter overall survival of ITD cases than wild-type cases (p value=0.0096).

For immunophenotypic analysis, this study demonstrated a consistent myeloid immunophenotype in adult AML with *FLT3* mutations, with expression of committed stem cell and myeloid antigens CD13, CD33, HLA-DR and MPO in almost all cases and CD64, CD117 and CD11c in more than 60% of cases, these data reliable with the previous Spanish study (166). However, some results discrepancy between this study and Spanish report. For example, Spanish results showed high expression of CD15 and CD14 in *FLT3*-ITD patients while this study showed low expression in mutant. This discrepancy may resulting from different nature, environment etc. This study showed more results than Spanish report because this study compared several types of *FLT3* mutation whereas Spanish study showed only results of *FLT3*-ITD. Both study also showed that FLT3-ITD patients did not display a unique immunophenotype suggesting that *FLT3* mutations does not represent a specific genetic lesion which had a characteristic phenotype as APL.

Finally, according to screening methods for *FLT3* mutations, this study suggest that *FLT3* mutations detection could easily be incorporated into the routine assessment of AML patients as the assay required only PCR, RFLP and gel electrophoresis. However, this traditional method had some disadvantages that should be of consideration. Bianchini *et al* showed that D-HPLC analysis of length mutations offer the advantage of higher sensitivity, especially for short insertion, because one patient who was identified as negative by PCR-gel electrophoresis was positive by D-HPLC (148). However, the increasing of gel percentage may help to increase sensitivity of

PCR-gel electrophoresis. Moreover, many recent studies also reported the other approaches which offered more advantages and higher sensitivity, including PCR/SSCP and NIRCA (146, 149). These approaches also provide the identification of a novel mutation not only in the part of exon 14 and exon 15 but also in other regions of *FLT3* gene. These observations suggest that additional investigation of the *FLT3* mutations may be of value for future studies of AML patients.

CHAPTER VII
SUMMARY

In this study, the part of exon 14 through exon 15 of *FLT3* gene was screened for *FLT3*-ITD mutations using standard PCR followed by gel electrophoresis assay. The coding region part of exon 20 of *FLT3* was also screened for *FLT3*-TKD mutation using PCR followed by RFLP. Sequencing method was used to clarify the mutations.

FLT3-ITD mutation was the most common in Thai AML patients followed by *FLT3*-TKD mutation. The dual mutation (ITD+TKD) were identified in some patients and each mutation occurred on a different allele.

All duplicated fragments were in-frame duplication in juxtamembrane region and sizes varied from 21 to 201 nucleotides (7 to 67 amino acids). All identified ITD included the duplication of at least one of SH2-binding motif (YFYV and/or YEYDLK).

A total of six *FLT3*-TKD mutations (seven of Asp835Tyr, four of Asp835His, one of each Asp835Glu, Asp835Ala, Ile836del and novel mutation) were identified in this study. The novel *FLT3*-TKD mutation, Asp835del+Ile836Val, also detected.

FLT3 mutations were mostly associated with a normal karyotype and a standard-risk patient. However, some cases were also found to coexist with primary and secondary chromosome aberrations such as t(8;21), t(15;17) and trisomy 8.

Patients with *FLT3* mutations had higher white blood cell counts and shorter overall survival than wild type *FLT3*. The immunophenotypic profile of patients with or without *FLT3* mutations did not differ.

FLT3 mutations are frequent in adult AML patients in Thailand. The prognostic impact of the *novel* TKD mutation remains to be determined in a larger population. The high prevalence of the activating *FLT3* mutations in Thailand and worldwide makes it a potential target for molecular-targeting therapy. Given the clinical efficacy of the selective tyrosine kinase inhibitor, imatinib mesylate, to

effectively treat BCR-ABL–positive chronic myeloid leukemia, it can be hoped that future research and development of novel *FLT3* inhibitory drugs should benefit AML patients with *FLT3* mutations, not only those patients in the developed western countries, but also Asian patients in this populated region of the world.

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APPENDIX

APPENDIX

1. Chemicals

Chemicals	Molecular weight (g/ml)	Source
Absolute ethanol (C_2H_2OH)	46.07	E.Merck, Darmstadtt, F.A., Germany
Absolute methanol (CH_3OH)	32.04	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.
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

2. Instruments

Applied Biosystems 377 DNA sequencer, Applied Biosystems, Foster, USA.

Gel documentation system, UVItec, Cambridge, UK.

Thermal cycler, Perkins Elmer PCR2400, 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

Proteiness K, Promega, Madison, USA.

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

5.3 10 mM Tris-HCl, pH 8.0

Tris base	12.11	g

Dissolved in distilled water and adjusted pH 8.0 with HCLl

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 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.

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

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

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

5.6 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.

5.7 2 mg/ml proteinase K in TE 20-5

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

The solution was mixed and immediately used.

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

Liquified phenol

Added 8-hydroxyquinoline to 0.1% w/v to a final conc. 0.1%

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

5.9 4 M NaCl solution

NaCl	23.38	g
Added distilled water to	100	ml

The solution was dispensed into aliquots and sterile by autoclaving

5.10 Chloroform-isoamyl alcohol (24:1)

Chloroform	21	volume
Isoamyl alcohol	1	volume

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

5.11 2 mM dNTPs solution (containing 2 mM dATP, dCTP, dGTP and dTTP), to prepare 100 µl, mix

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

The solution was kept at -20 °C.

5.12 6x gel loading buffer

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

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

BIOGRAPHY

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