FLUORESCENCE IN SITU HYBRIDIZATION (FISH) TECHNIQUE FOR DETECTION OF GENETIC ABNORMALITIES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

In this study, fluorescence *in situ* hybridization (FISH) technique using DNA probe specifically for TEL/ AML1, ETV6(TEL), TCF3/ PBX1, TCF3, BCR/ ABL, MLL, IGH, and PAX5 genes was performed to detect gene rearrangements and frequent genetic lesions in 32 pediatric acute lymphoblastic leukemia (ALL) patients.

Chromosomal aberrations shown by FISH positive technique were detected in 27 (84.4%) of all pediatric ALL samples. These included 10 cases (31.2%) of TEL/AML1 fusion, 1 case (3.1%) of TCF3/PBX1 fusion, 2 cases (6.2%) of BCR/ABL fusion, 1 case (3.1%) of MLL split signal, 3 cases (9.4%) of IGH split signal, 1 case (3.1%) of PAX5 split signal, and the gain or loss, with or without translocation of these genes were also observed.

To summarize, FISH technique using specific DNA probes is a powerful tool to be used as a routine procedure for the detection of genetic abnormalities in newly diagnosed ALL patients. The difference in chromosomal aberrations could be used as a marker for the prognosis and treatment outcome of pediatric ALL patients.

KEY WORDS: ACUTE LYMPHOBLASTIC LEUKEMIA / FLUORESCENCE IN SITU HYBRIDIZATION

92 pages

การตรวจหาความผิดปกติของยีนในผู้ป่วยเด็กมะเร็งเม็ดเลือดขาวชนิดลิมฟอยด์แบบเฉียบพลัน โดยวิธีฟลูออเรสเซนส์อินไซตูไฮบริไดเซชั่น

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บทคัดย่อ

วัตถุประสงค์ของวิทยานิพนธ์ เพื่อใช้เทคนิค ฟลูออเรสเซนส์อินไซตูไฮบริไดเซชั่น ในการตรวจคัดกรองหายีนลูกผสมและยีนที่มีความผิดปกติอื่นๆ ที่มีความจำเพาะต่อการเกิดโรคมะเร็งโลหิตขาวชนิดลิมฟอยด์แบบเฉียบพลันในเด็ก จากตัวอย่างไขกระดูกจำนวน 32 ตัวอย่าง ผลการทดสอบพบผู้ป่วยที่มีความผิดปกติของยีนจำนวน 27 ราย (84.4%) ได้แก่ ยีนลูกผสมชนิด TEL/ AML1 จำนวน10 ราย (31.2%), TCF3/ PBX1 (3.1%) จำนวน 1 ราย (3.1%), ชนิด BCR/ ABL จำนวน 2 ราย (6.2%), ชนิด MLL จำนวน 1 ราย (3.1%), ชนิด IGH จำนวน 3 ราย (9.4%), และ ชนิด PAX5 จำนวน 1 ราย (3.1%) นอกจากนี้ ความผิดปกติอื่นๆ เช่น การขาดหายหรือเพิ่มขึ้นของยีน โดยอาจพบร่วมกับยีนลูกผสมอื่นๆ ก็พบได้เช่นกัน

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LIST OF ABBREVIATIONS

%	=	Percent
μ	=	Micro (10^{-6})
°C	=	Degree of celcius
ALL	=	Acute lymphoblastic leukemia
AML	=	Acute myeloid leukemia
bcr	=	Break point cluster region
CD	=	Cluster designation
CNS	=	Central nervous system
DAPI	=	4',6-diamidino-2-phenylindole
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylene diamine tetraacetate
FAB	=	French-American-British
FISH	=	Fluorescence *in situ* hybridization
g	=	Gram
Hb	=	Hemoglobin
HCl	=	Hydrochloric acid
HLA-DR	=	Human leukocyte antigen subclass DR
IgH	=	Immunoglobulin heavy chain
KCl	=	Potassium chloride
m	=	Milli (10^{-3})
M	=	Molar
mg	=	Milligram
$MgCl_2$	=	Magnesium chloride
min	=	Minute
MLL	=	Mix lineage leukemia
MRD	=	Minimal residual disease
mRNA	=	Messenger ribonucleic acid

LIST OF ABBREVIATIONS (cont.)

NP-40	=	Nonidet P40
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
pH	=	Power of hydrogen ion
RNA	=	Ribonucleic acid
RNase	=	Ribonuclease
rpm	=	Revolution (s) per minute
RT	=	Reverse transcription
SD	=	Standard deviation
sec	=	Second (s)
SSC	=	Standard saline citrate
TdT	=	Terminal deoxynucleotide transferase
U	=	Unit (s)
WBC	=	White blood cell

CHAPTER I
INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy, accounting for 75% to 80% (1) and approximately 80–85% are B-ALL phenotype (2). B-lineage ALLs can be further subclassified as early pre-B, pre-B, transitional pre-B, or B-cell (3). Clinically important molecular alterations in B-precursor ALL include the translocation t(12;21)(p13;q22); TEL-AML1 (or ETV6/ CBFA2), t(1;19) (q23;p13.3); E2A-PBX1 and t(9;22)(q34;q11.2); BCR-ABL. Other chromosomal regions, such as 11q23; MLL, 14q32; IGH and 9q11; PAX5 undergo translocations, deletions and inversions with numerous partners gene (Table 1.1) (4).

Table 1.1 Common genetic abnormalities in childhood ALL

Phenotype	Chromosomal Abnormalities	Genes		Prognosis
Early Pre-B	Hyperdiploidy (50-60 chromosomes)			Good
	t(12;21)	TEL	AML1	Good
Pre-B	t(1;19)(q23; p13)	PBX	E2A	Intermediate
	t(9;22)(q34;q11) (Philadelphia chromosome)	ABL	BCR	Poor
Mature B cell	t(8;14)(q24; p32)	MYC	IGH	Poor
	t(2;8)(q12; p24)	IGK	MYC	Poor
	t(8;22)(q24; p11)	MYC	IGL	Poor
Infant leukemia	t(4;11)(q21; p23)	AF4	MLL	Very poor

Numerous treatment protocols for hematologic disorders require quantitative procedures to access responsiveness to therapy. This can be done by using various genetic tests including quantitative cytogenetic studies, fluorescence *in situ* hybridization (FISH) (5), southern blot analysis (6), and reverse transcriptase polymerase chain reaction (RT-PCR) (7). No single genetic testing procedure fulfills all the needs of clinical care for patients with hematologic disorders. Thus, combination of testing methods that are both accurate and cost-effective for each clinical situation has become important strategy. However, some hematologic specimens are not available for conventional cytogenetic studies whereas FISH studies of interphase nuclei can be performed on a variety of specimens, including fixed cells from peripheral blood, bone marrow and paraffin embedded specimens. Furthermore, FISH is also useful to detect abnormalities when the results of conventional cytogenetic studies are inconclusive because of cryptic or masked translocation. In 2003, ZHANG Lijun, *et al.*, tried to detect chromosome changes related to prognosis of patients with ALL by using a combination of multiple FISH probes (8). In 2008, Oskar Hass described FISH probes for screening about ETV6, MLL, BCR/ABL, E2A, IGH, PAX5 and p16 genetics abnormalities which could be found about 80% of B-cell precursor (BCP) ALL patients of St Anna Children's Hospital Vienna, Austria (9). Thus, this study aims to detect the most common structural chromosome aberrations by using FISH analysis with three translocation probes; TEL/AML1, BCR/ABL, TCF3/PBX1 and five break apart probes; MLL, IGH, ETV6, TCF3, PAX5 in 32 Thai childhood ALL patients. These results could support the traditional diagnostic methodologies to accurate assignment of a substantially higher percentage of patients to the appropriate treatment protocols.

CHAPTER II
OBJECTIVE

1. To detect chromosomal aberrations in pediatric acute lymphoblastic leukemia (ALL) patients by fluorescence *in situ* hybridization (FISH).
2. To use difference chromosomal aberrations as a marker for the prognosis and treatment of pediatric acute lymphoblastic leukemia (ALL) patients.

CHAPTER III
LITERATURE REVIEW

Acute lymphoblastic leukemia (ALL) is mainly a disease of childhood and young adult with a peak incidence at 4-5 years of age, and another peak in old age. It is characterized by uncontrolled precursor lymphocyte proliferation, survival, and blocked differentiation that initiates from the bone marrow, thymus, and perhaps fetal hemogenic organs. ALL causes damage and death by crowding out normal cells in the bone marrow and by spreading (metastasizing cell) to other organs. The overall cure rate, in 85% of children, have long-term disease-free survival (10). Accurate morphologic, cytochemical, immunophenotypic, and genetic data are critical for guiding treatment decisions.

3.1 Pathogenesis

ALL develops as the result of a combination of genetic and environmental factors. Some of these factors are well established, e.g. genetic predisposition (including certain genetic conditions, such as Down's syndrome and Fanconi's anemia), viral infection (HTLV-1), ionizing radiation, chemical agents (benzene), drugs (alkylating agents, topoisomerase II inhibitors). However, the link between a certain known predisposing agent and development of ALL can only be shown in a small proportion of cases and in the majority the question of the cause of ALL remains unresolved. Genetic studies of identical twins, detection of leukemia-specific fusion genes or immunoglobulin genes rearrangements in neonatal blood have established the prenatal origin of leukemia in many childhood cases (11, 12). It is believed that the first genetic event necessary for the development of childhood ALL frequently originates *in utero*, and is followed by additional postnatal mutations that lead to clinically overt leukemia (Figure 3.1) (13-15).

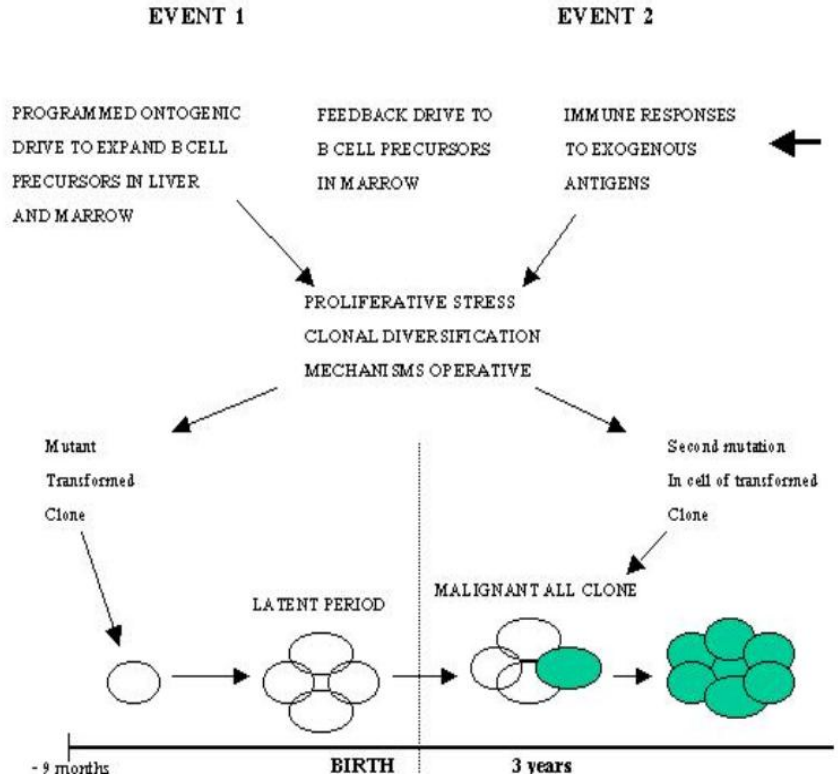

Figure 3.1 Greaves' two-hit model for the leukemogenesis of childhood ALL

3.2 Classification of acute lymphoblastic leukemia

Acute lymphoblastic leukemia are currently classified on the basis of the cell of origin. At diagnosis cytochemical, immunophenotypic, cytogenetic, and occasionally ultrastructural and molecular genetic studies are used to fully characterize the leukemic blasts and assign a diagnostic category as summarized in Tables 3.1. Better characterization of these heterogeneous neoplasms will ultimately lead to further understanding of the pathogenesis of leukemia (16).

Table 3.1 Blast cell characteristics of acute lymphoblastic leukemia subtypes

	Early Pre-B Cell	**Pre-B Cell**	**B Cell**	**T Cell**
Gene rearrangement				
Heavy chain	+*	+	+	-
Light chain	±	±	+	-
Immunologic features				
Cytoplasmic μ	-	+	-	-
Membrane Ig	-	-	+	-
CD10 (CALLA)	+	±	±	±
Ia/ HLA-DR	+	+	+	-
CD24, CD19, CD20	+	+	+	-
CD2,CD5,CD7	-	-	-	+
Cytochemical features				
Nuclear TdT	+	+	-	+
5'- Nucleotidase	+	+	-	-
Acid phosphatase	-	-	-	+
Cytogenetic abnormalities	t(12;21)	t(1;19)	t(8,14), t(2;8), t(8;22)	t(1;14), t(11;14), del(9p)
Morphology				
FAB L1	90%	90%	10%	95%
FAB L2	10%	10%	15%	5%
FAB L3	0	0	75%	0

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

3.3 Laboratory diagnosis methods

3.3.1 Morphology

Subtyping of the various forms of ALL used to be done according to the French American British (FAB) classification. FAB group described three types of ALL (L1, L2, and L3) which are distinguished on the basis of microscopic appearance of the leukemic cells, as seen on Wright-Giemsa–stained smears (Table 3.2) (17, 18). Most commonly, L1 blasts are small to intermediate in size, scanty cytoplasm, condensed nuclear chromatin, and indistinct or absent nucleoli. Less commonly, L2 cells may be larger, with moderate amounts of pale basophilic cytoplasm, finely dispersed nuclear chromatin, and prominent nucleoli. Very rarely, L3 cell consists of large blasts, with abundant deeply basophilic and occasional vacuolated cytoplasm, coarsely clumped nuclear chromatin, and variably prominent nucleoli.

Table 3.2 FAB classification of acute lymphoblastic leukemia

Morphology	FAB L1	FAB L2	FAB L3
Cell size	Small Homogeneous	Large Pleomorphic	Large Homogeneous
N/C ratio	Higher in > 75% of cells	Lower in > 25% of cells	Variable
Nuclear shape	Regular, may have cleft or indentation	Irregular, may have cleft or indentation	Regular, oval to round
Nucleoli	Indistinct or notvisible	One or more per cell; large, prominent	One or more per cell; large, prominent
Cytoplasmic basophilia	Moderate	Moderate	Deep
Cytoplasmic vacuoles	Not prominent	Not prominent	Sharply defined

N/C: nuclear-to-cytoplasmic ratio

3.3.2 Cytochemistry

Cytochemical stains are used to differentiate acute myeloid leukemia (AML) from ALL. The leukemic cells of ALL are uniformly negative for myeloperoxidase (MPO), Sudan black B (SBB), and lack the characteristic NaF-sensitive α-naphthyl acetate esterase (ANAE) of monoblasts. Cytochemical tests are supplemented by immunophenotyping and electron microscopy if necessary (19).

The myeloperoxidase reaction is strongly positive in cells of the granulocytic series but weakly positive in monocytes. The reaction may be positive in undifferentiated myeloblasts that lack of azurophilic granules on Wright's stain (AMLM1). Peroxidase are negative in lymphocyte and erythroid precursor. Myeloperoxidase positive blasts have been detected by electron microscopy in approximately 15% of ALL (20). Most of these patients have high-risk ALL, with a predominance of T-cell phenotype, and a long-term poor prognosis on conventional anti-ALL therapy. Which suggest that these patients may be benefit from intensification treatments with drugs effective against AML.

Sudan black B stains a variety of lipids (phospholipids, sterols, and neutral fat). The pattern of staining closely parallels to peroxidase; Sudan black B may be more sensitive in some leukemic blasts that are myeloperoxidase negative and Sudan black B weakly positive. Sudan black B is very useful in distinguishing AML from ALL, although a few well-documented cases of Sudan black positive ALL have been reported (21). Auer rods are intensely pseudophilic. PAS staining reacts primarily with glycogen. ALL lymphoblasts often have prominent PAS staining in the form of coarse granules.

3.3.3 Immunophenotype

Immunophenotyping makes the blast cell possible to identify specific lineage and stage of differentiation. The development of antibodies directed against hematopoietic and lymphoid cell antigens had greatly facilitated the diagnosis and classification (Table 3.3). Antibodies are assigned cluster designations (CD) by International Workshops on leukocyte differentiation antigens on the basis of their reactivity with standard panel of cells (22). The first generation of surface marker tests led to the recognition of three broad classes of ALL : T cell, B cell, and non-T, non-B

ALL. The latter class was divided further into groups that either expressed or did not express the common ALL antigen (CALLA, CD10) and were called CALLA positive ALL and null-cell ALL, respectively (23). Subsequent investigations led to recognition of early pre-B and pre-B-cell ALL and to appreciation for the heterogeneity of T-cell ALL (24).

Tables 3.3 Immunologic markers used in the acute leukemia classification

Lineages	Antigens
B cell	CD19, CD20, CD21, CD22, CD23, CD24
T cell	CD1, CD2, CD3, CD4, CD5, CD7, CD8
Lymphoid	TdT
Myeloid	CD13, CD33, CD11b, CD15,
Monocytic	CD14, CD11b
Erythroid	Glycophorin A
Megakaryocyte	CD41, CD42b, CD61
Lineage Independent Antigens	
HLA-DR	HLA class II
CD45	Leukocyte common antigen
CD34	Stem cell antigen
CD10	Common ALL antigen (CALLA)

3.3.3.1 B-Lineage ALL

The frequency of B-cell-specific or B-cell-associated antigen expression in ALL is summarized in Table 3.4 (25). HLA-DR is a sensitive marker of B lineage, but it lacks specificity. More than 99% of ALL expressing the HLA-DR positive, CD5 and CD7 negative phenotype were B cell origin when studied with a comprehensive panel of antibodies. CD19 and CD24 are nearly as sensitive as HLA-DR but more specific. CD20 and CD22 are highly specific but less sensitive. Expression of CD20 in leukemic blasts is usually associated with CD19. CD10 (CALLA) is expressed by approximately 90% of B-lineage ALL blasts (26). The definition of distinctive subgroups of B-lineage ALL is complicated by the observation that in nearly one half to two thirds of patients, expression of immunophenotypes is asynchronous with the stages of normal lymphoid differentiation (27). The immunophenotypic subtypes of B-lineage ALL do not conform in all respects to the pattern of B cell differentiation outlined in Figure 3.2.

- Early Pre-B-cell ALL

The most primitive B-lineage ALL blast lacks cytoplasmic and surface immunoglobulin and cytoplasmic heavy μ chains. They are recognized by expression of one or more antigens expressed by early B progenitors (CD19, CD24) (28) or by commitment to immunoglobulin synthesis as determined by heavy chain gene rearrangement. This type of ALL accounts for more than 70% of cases in children and approximately 50% of cases in adults. Ninety precent of children with early pre-BALL express CD10. In earlier classifications this was referred to as CALLA positive or “common” ALL. Both CD10 and CD34 impact favorably on prognosis (29). Characteristically, both TdT and 5’-nucleotidase activities are increased.

- Pre-B-cell ALL

Pre-B-cell ALL blasts are characterized by cytoplasmic heavy chains (cμ) in the absence of light chains or surface immunoglobulin (sIg) (30). Ninety percents or more are CD10-positive and most are classified as FAB L1. They are TdT, HLA-DR, CD19, CD20, and CD24-positive.

- Transitional Pre-B ALL

A small subgroup of ALL (<1%) patients has blasts that express cytoplasmic μ and surface μ but not light chains, suggesting these cells are in transition between the pre-B and B stages of differentiation (31). This type of ALL is associated with lower leukocyte counts and a higher frequency of DNA indexes greater than 1.16 when compared with pre-B ALL. Patients lack bulky extramedullary disease and chromosomal translocations involving chromosome 8 as seen in B-ALL. The 4-year relapse-free survival appears better than that for children with pre-B ALL but the difference is not statistically significant.

- B-cell ALL

Approximately 1 to 2% of blast cell in patients with ALL express surface immunoglobulin, usually IgM 32). The cells are HLA-DR, CD19, CD20, CD24-positive; CD10 and TdT expression are often lost, B-cell ALL cells in approximately 75% of patients share morphologic (FAB-L3) and cytogenetic similarities with Burkitt's lymphoma (32). The aggressive clinical course associated with this subtype is consistent with a leukemic phase of Burkitt's lymphoma (33). The subset of B-ALL without L3 morphology is TdT negative and has a predominance of λ light chain expression. Patients are older males with extensive marrow and blood involvement and short median survival (5.5 months) (34).

3.3.3.2 T-Lineage ALL

Approximately 15% of ALLs have a T-cell phenotype. Monoclonal antibodies directed against T-cell antigens have greatly improved our ability to identify T-cell lineage and have led to an appreciation for the immunologic heterogeneity of T-cell malignancies (35). In as many as one third of cases of T-ALL, the cells are E-rosette (CD2) negative. Blasts in some patients with T-cell ALL are CD7 negative, diagnostic evaluation should include the use of monoclonal antibodies to CD5 and CD2 (36). CD2 and CD7 are expressed in AML in 5 to 60% of cases, again emphasizing that antigen expression must be interpreted in the context of a panel of antibodies (37). Most cases of T-ALL are HLA-DR negative. The presence of

cytoplasmic CD3 in the absence of surface CD3 is highly specific for T-ALL, but may be seen in normal and neoplastic natural killer cells (38).

Many T-cell ALL blasts express a combination of antigens not found in normal T-cell progenitors. Leukemic cells that are CD7 positive but CD4 and CD8 negative are capable of multilineage differentiation *in vitro* (39). Patients with this immunophenotype respond poorly to chemotherapy for either ALL or ANLL. In contrast to B-cell ALL, in which subset designation has clear clinical and therapeutic implications, T-cell ALL subset classification appears to be of limited clinical significance. Most T-cell malignancies corresponding to the prothymocyte stage are characterized clinically by acute leukemia, whereas those corresponding to the more mature stage of differentiation have either a leukemic or a lymphoma phenotype (40).

Table 3.4 Relative frequency of B-lymphoid antigen expression in B-lineage acute lymphoblastic leukemia

Antigen	Cases Positive (%)
La/HLA-DR	99.8*
CD19	98
CD24	97
CD10 (CALLA)	92
CD19	90
CD22	74
CD34	64
CD20	36
CD21	4

*Positivity defined as antigen expression by 20% or more of leukemic blast cells. (Adapted from Borowitz MJ. Immunological markers in childhood acute lymphoblastic leukemia. Hematol Oncol Clin North Am 1990; 4:743-65)

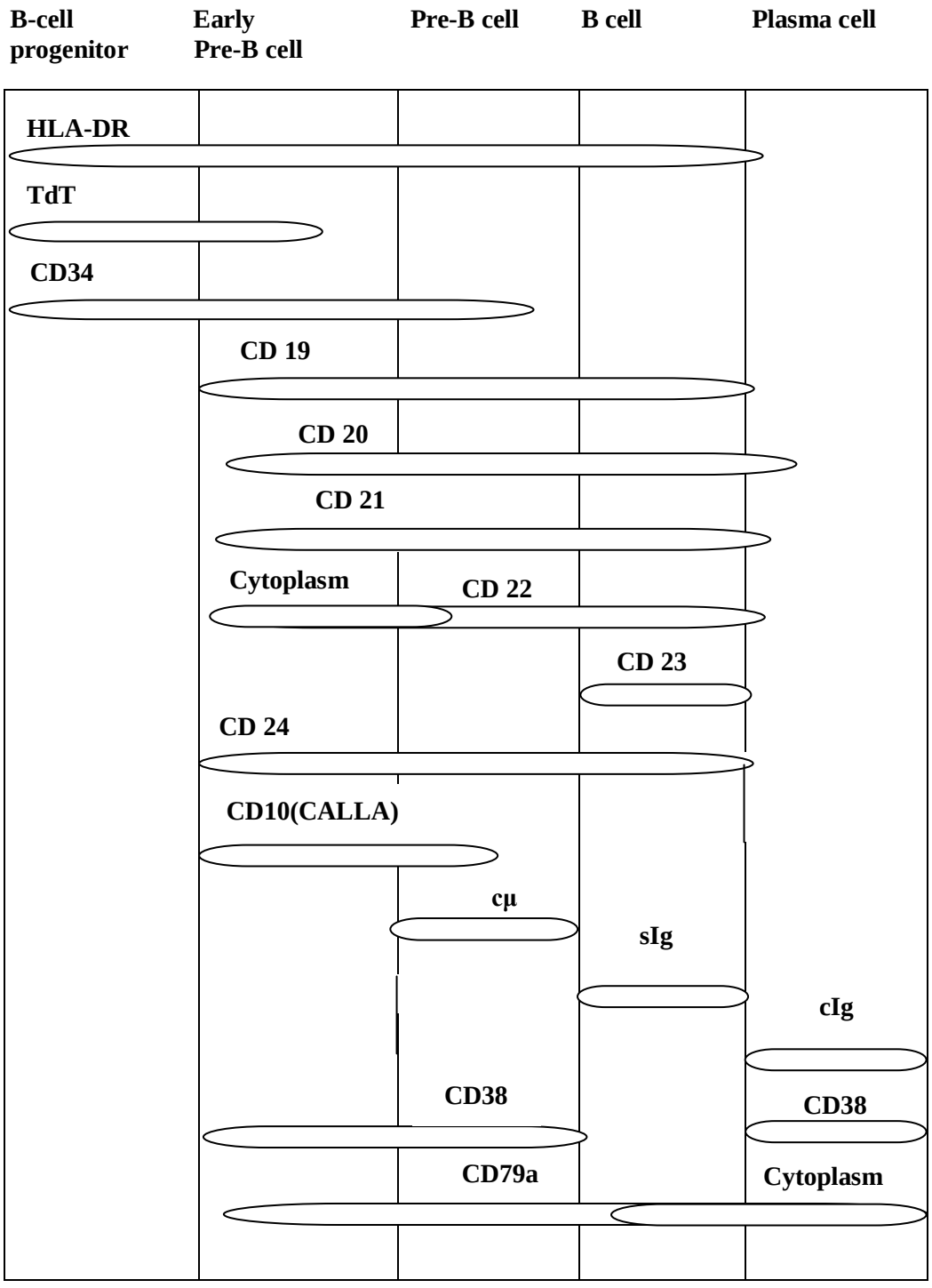

Figure 3.2 Changes in antigen expression of B-lineage cells during normal maturation. Five stages of maturation are defined by more or less distinctive combinations of antigen expression. In B-lineage ALL, expression of these antigens often is asynchronous. Cytoplasmic immunoglobulin (cIg); TdT, terminal deoxynucleotidyl transferase; slg, surface immunoglobulin.

3.3.4 Chromosome abnormalities

Cytogenetic abnormalities of leukemic cells can be characterized as changes involving chromosome number (ploidy) or chromosome structure (translocation, inversion, etc.). Ploidy is determined either by quantitation of the modal number of chromosomes in metaphase preparations or by flow cytometry. Karyotypic analysis and flow cytometry provide complementary data. Many of the breakpoints involved in chromosomal rearrangements are located at sites of oncogenes (41) Especially in ALL, the distribution of the abnormalities is clearly different in children and adults. In addition, the distributions in infants and older children differ remarkably from each other and which about half of all childhood ALL cases having recurrent translocations (Figure 3.3) (Table 3.5) (42). Present diagnostic methods reveal genetic aberrations in about 90% of ALL and AML patients. In most cases an aberration, which has been found at the time of diagnosis, is tightly connected with a specific leukemia type (43).

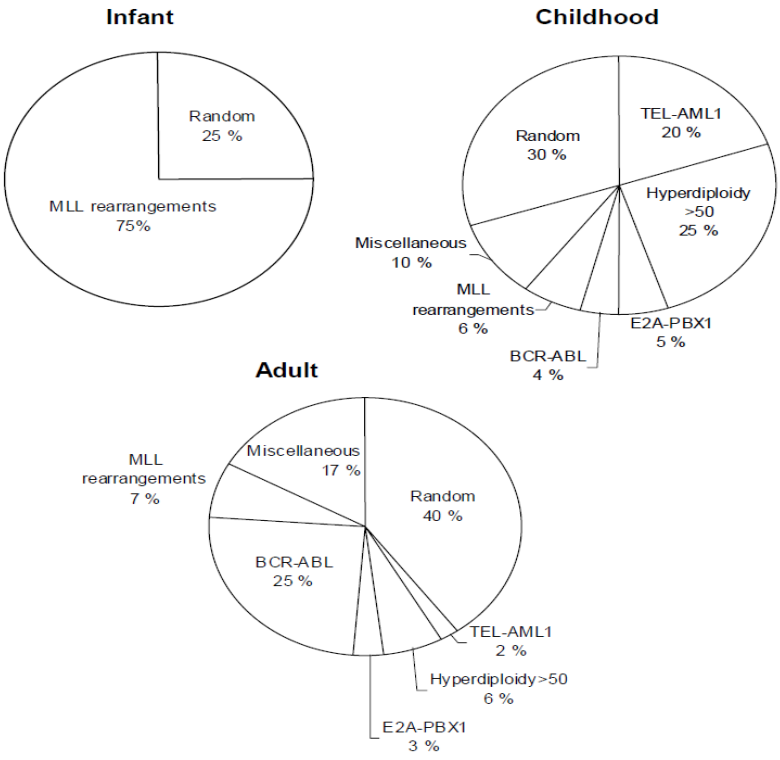

Figure 3.3 Prevalence of genetic changes in ALL with respect to different age groups.

Table 3.5 Common genetic alterations in childhood ALL

Karyotype	Molecular alteration	Frequency
B-precursor ALL		
t(12;21)(p13;q22)	*TEL-AML1*	25%
t(1;19)(q23;p13)	*E2A-PBX1*	6%
t(9;22)(q34;q11)	*BCR-ABL*	4%
t(4;11)(q21;q23)	*MLL-AF4*	4%
Other 11q23	Other *MLL* fusions	
Hyperdiploid (DI≥1.16)	Unknown	20%
B cell ALL		2%
t(8;14)(q24;q32)	*MYC* dysregulation	
t(2;8)(q12;q24)	*MYC* dysregulation	
t(8;22)(q24;q11)	*MYC* dysregulation	
T cell ALL		8%
t(1;14)(p32;q11)	*TALI* dysregulation	
t(1;7)(p32;q35)	*TALI* dysregulation	
t(7;9)(q34;q32)	*TAL2* dysregulation	
t(7;19)(q34;p13)	*LYL1* dysregulation	
t(10;14)(q24;q11)	*HOX11* dysregulation	
t(7;10)(q35;q24)	*HOX11* dysregulation	
t(11;14)(p15;q11)	*LMO1* dysregulation	
t(7;11)(q35;p13)	*LMO2* dysregulation	
t(11;14)(p13;q11)	*LMO2* dysregulation	
t(1;7)(p34;q34)	*LCK* dysregulation	
t(7;9)(q34;q34)	*TAN1* dysregulation	

3.3.4.1 Genetic methods used in the diagnosis

The nomenclature of chromosomal aberrations has evolved throughout the years and is updated regularly by the International Standing Committee on Human Cytogenetic Nomenclature (ISCN); the most resent update occurred in 1995 (44).

Cytogenetic and molecular analyses are essential for the classification of hematologic malignancies. Chromosome abnormalities in ALL are found in 65-70% of the patients by standard karyotyping (45). Using molecular as well as cytogenetic studies, greater than 90% of ALL have abnormalities (46, 47). All leukemia cases should have an adequate cytogenetic analysis, and in 80–90% of leukemia a clonal chromosomal abnormality should be identified (48). Moreover, genetic rearrangements should be detectable in most leukemia patients with the help of fluorescence *in situ* hybridization (FISH) and other molecular techniques providing the availability of appropriate DNA probes (49). They are important indicators of prognosis and may be divided into two groups: numerical and structural abnormalities.

3.3.4.1.1 Conventional cytogenetic

Conventional cytogenetic had been used in the diagnosis of leukemia since 1970's (50). It is based on the recognition of metaphasic chromosomes according to their specific banding patterns. The most widely used cytogenetic staining is Giemsa- or G-banding. Standard cytogenetic is still an important routine method at the diagnostic stage of leukemia, giving an overview of genetic alterations in malignant cells. The method has, however, several weaknesses. The lack of metaphase may be a problem because the leukemic cells, especially the blasts of childhood ALL, often fail to proliferate in the cell culture. Other drawbacks of cytogenetic method are that the quality is often insufficient for detailed analysis or the aberration is too complex to be interpreted. Furthermore, many important chromosomal aberration are missed. For example the translocation t(12;21) of ALL cannot be detected by the method because the aberration does not change the banding pattern or the size of the chromosomes involved (Figure 3.4). Finally, conventional cytogenetic test is laborious and time-consuming, and only about twenty cells can be analysed per patient. This is also the reason why this method cannot be used for the detection of minimal residual disease in patient follow-up.

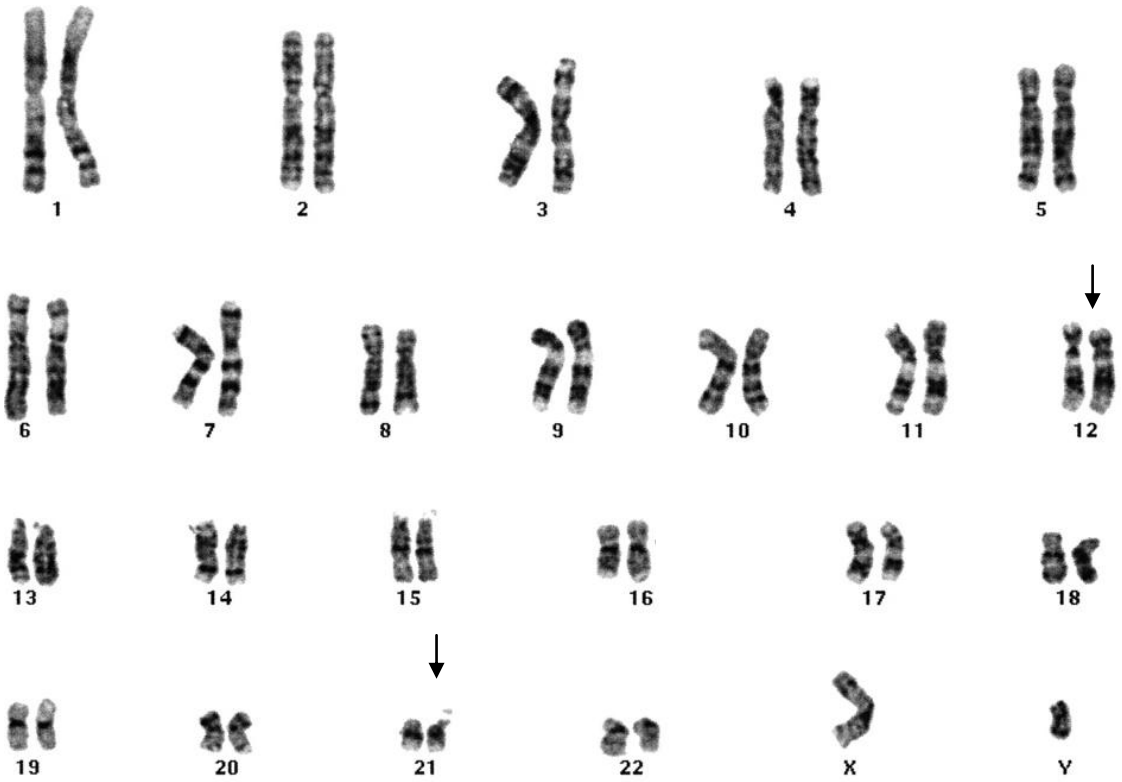

Figure 3.4 Partial GTG-banding karyotype showing t(12;21) (a) Partial FISH analysis showing the AML1 hybridization signals on derivative chromosomes 12 and 21, and on the normal chromosome 21 (b)

3.3.4.1.2 Molecular genetic methods

A specific gene fusion caused by a translocation can be detected by Southern blotting and polymerase chain reaction (PCR). In Southern blotting, DNA is cut by sequence-specific restriction enzymes and the resulting fragments are separated in gel electrophoresis. The size of the fragment recognized by a gene specific probe is different in the fusion gene as compared to the fragment in the normal gene.

The weaknesses of the method are that it is slow and required large amount of cells. In most translocations the variance in cleavage sites is so great that PCR cannot be performed on DNA. RNA is therefore the usual source of PCR to detect gene arrangements. First RNA has to be transformed into more stable complementary DNA (cDNA) by reverse-transcriptase (RT) enzyme and, accordingly,

this type of PCR is called RT-PCR. Two specific primers, one for each side of the fusion point are needed for the reaction. No PCR product is detected in samples without a gene fusion, whereas cDNA is amplified exponentially in samples that contain the fusion. Southern blotting and RT-PCR are used as a support for diagnostics based on the result of G-banding analysis. Because specific probes or primers are required, the methods can be routinely used only for the most well-defined leukemia-specific translocations, such as t(12;21), t(9;22), t(1;19) and t(4;11) in ALL. RT-PCR is also widely used to monitor minimal residual disease. The detection of the rearrangement from RNA requires that specific primers are designed for each patient. The task is laborious and interpretation of the results is often difficult. In conventional PCR, contamination can be a recurrent problem. In addition, the method is not quantitative, which makes it deficient especially in residual disease monitoring. These drawbacks are overcome in quantitative real-time PCR, performed in a closed system, in which a fluorescent signal is generated and detected continuously during the amplification. The extent of fluorescence is directly proportional to the extent of PCR product. Real-time PCR has already been reported to be applicable to the quantification of residual disease in childhood ALL (51-53).

3.3.4.1.3 Molecular cytogenetic methods

Molecular cytogenetics refer to the study of DNA or gene at chromosome or cell level. It is based on *in situ* hybridization (ISH), in which a DNA probe binds with a specific region in the genome of the cell. The detection is based either on a colour produced by enzymatic reaction or on fluorescence, the latter being more widely used. In fluorescence *in situ* hybridization (FISH), the probe is labelled with fluorochrome and the detection is performed using fluorescence microscope with specific filters (54).

- **Chromosome painting and multicolour-FISH**

In chromosome painting, the whole chromosome or a chromosome arm is stained. The analysis is made from metaphase cells. The whole chromosome painting probe is a mixture of fragments from the entire length of a specific chromosome. Painting probes can be derived from chromosome-specific libraries, by

PCR amplification from chromosome fractions or from microdissected DNA specific for each chromosome. Chromosome painting is used to verify and define translocations or numerical aberrations seen in G-banding. However, the ability of chromosome painting to detect translocations like t(12;21) that are located near the telomeric regions of the chromosomes is limited (55). In multicolour-FISH alias 24-colour FISH the whole set of chromosomes is painted in a single hybridization. Each chromosome is stained with a different combination of five fluorochromes. Using appropriate filters and software, all the chromosomes can be visualized in different artificial colours, each chromosome showing a specific colour. Multicolour-FISH is called multiplex FISH (M-FISH) (56) or spectral karyotyping (SKY) (57) depending on the technical details applied to fluorescence detection. Like the G-banding method, multicolour-FISH gives an overview of chromosomal changes in the whole of the genome. It is especially useful in defining complex translocations and so-called marker chromosomes with unknown origin, and it is used to support standard cytogenetics in diagnosis (55, 58, 59).

- **Interphase-FISH**

There are two main approaches of FISH probe design for use on (interphase) nuclei, that is, fusion-signal FISH and split-signal FISH. The classical fusion-signal FISH approach uses two differentially labeled probes, red and green, which flank the breakpoint regions of the two genes, which are involved in the translocation (Figure 3.5a). In normal karyotypes, that is, without chromosome aberration, two red signals and two green signals are detectable. In case of a translocation, a red and a green signal will be juxtaposed giving rise to a colocalized green/red signal, which will generally appear as a yellow signal. In addition, separate green and red signals of the unaffected chromosomes will be visible. The split-signal FISH approach also uses two differentially labeled probes, but these probes are located in only one of the two involved genes, here after called the target gene, and are positioned at opposite sides of the breakpoint region of the target gene (60,61) (Figure 3.5b). In normal karyotypes, two colocalized green/red signals usually appearing yellow will be visible. A translocation will result in a split of one of the colocalized

signals, resulting in a separate green and red signal together with a fused signal of the unaffected chromosome (60,61).

The study of interphase cells has major advantages over the study of mitotic cells. As mentioned, especially the leukemic blasts of ALL proliferate poorly in cell culture, and mitotic cells may not represent the neoplastic clone (55). Another advantage of interphase FISH is that it can be combined with the study of the immunophenotype and morphology of the cell by immunocytochemical staining (62). This makes it possible to define the lineage and maturation stage of the cells with the aberration. Finally, interphase FISH can be used for stored material including smear preparations, which is beneficial especially in retrospective studies (63). Interphase-FISH can be used both in diagnosis and follow-up.

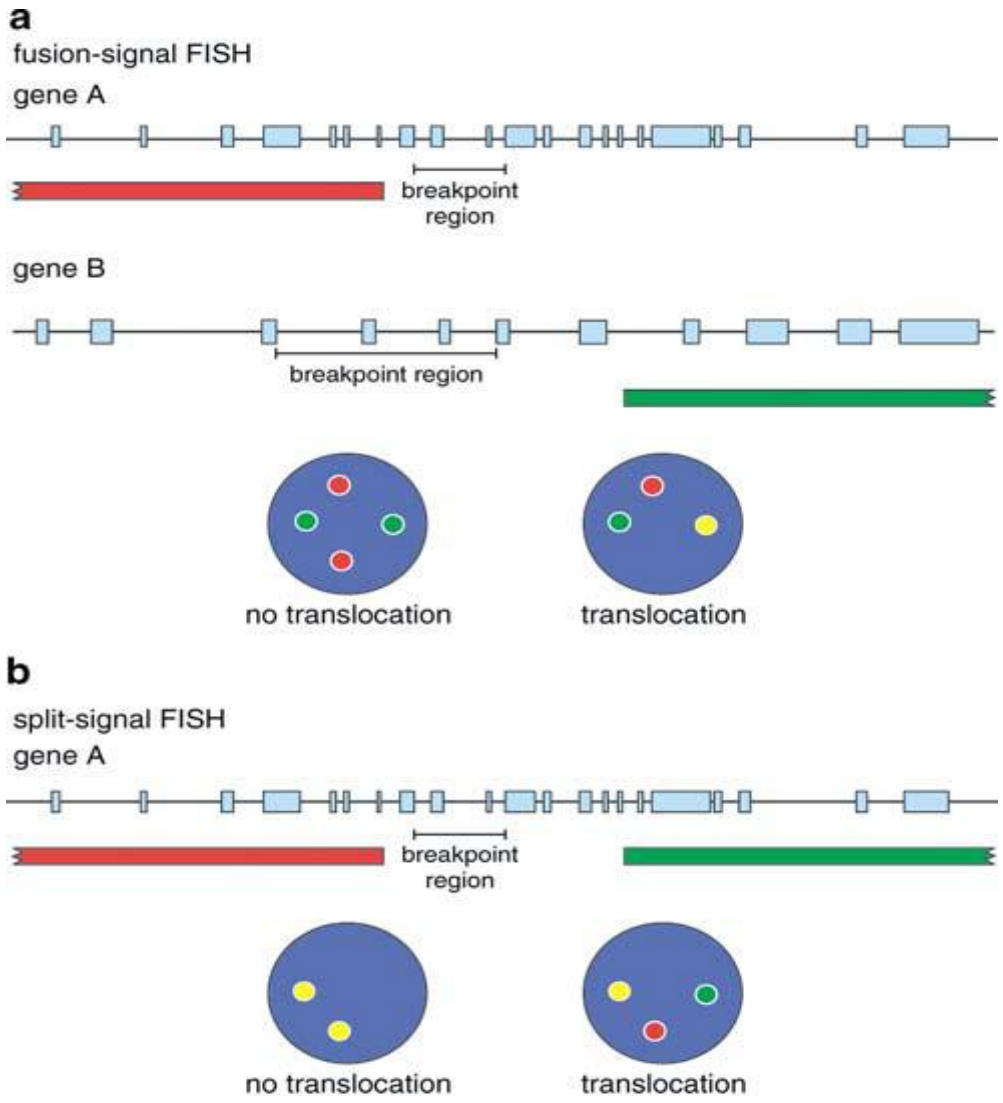

Figure 3.5 Differences between fusion-signal FISH and split-signal FISH. (a) Fusion-signal FISH with two probes located in the two genes, which are involved in the chromosome translocation. In normal situations, two green and two red signals will be present. In case of a translocation, a green and a red signal co-localize generally appearing as a yellow signal together with the separate green and red signals of the unaffected genes. (b) Spit-signal FISH with two probes positioned at opposite sides of the breakpoint region in one gene, which is involved in the chromosome translocation. In normal situations, two yellow signals will be present, while in case of a translocation separate green and red signals will be present together with the colocalized signal of the unaffected gene.

- **Comparative genomic hybridization**

Comparative genomic hybridization (CGH) (64) is a modification of FISH that allows screening of the whole genome for DNA sequence gains and losses in a single experiment (58, 65, 66). The principle of the method, the DNA extracted from a patient sample is cut into small fragments and labelled with green fluorochrome using nick translation. Similarly, DNA extracted from normal tissue, usually blood, is cut and labelled, but red fluorochrome is used for labelling. Equal amounts of differently labeled patient DNA and normal DNA are mixed and hybridised into normal metaphase spreads. The patient DNA and normal DNA compete for the same target sequences, and the hybridization takes place proportionally to the amounts of patient DNA and normal DNA present. Consequently, the over represented chromosomal regions in the patient are seen as green, and under represented as red. For a detailed and reliable analysis, images of the hybridized metaphases have to be captured by a CCD (charge coupled device) camera connected to a fluorescence microscope, and the intensities of red and green fluorescence are calculated using image analysis software. The software produces profiles of green-to-red fluorescence ratios along the entire length of each chromosome. The gains and amplifications of patient DNA can be detected as chromosomal regions with increased ratio, and the losses as regions with reduced ratio.

- **Gene expression profiling by DNA arrays**

The sequencing of human genome has identified approximately 19,000 genes and tens of thousands partial fragments of genes termed expressed sequence tags (ESTs) (67). At present, the number of human genes is estimated to be about 30,000–35,000 (68, 69). The availability of the sequences has made it possible to use novel techniques to study the function of genes as well as their roles in diseases. The most powerful of these techniques is DNA array technology, first introduced in 1995, which has made it possible to study the expression of thousands genes in a single experiment (70-72). Limitation of microarray is prohibitively expensive to perform sufficient replicates and other controls, and thus experiments lack statistical power(62).

3.4 Clinical characteristics of ALL

3.4.1 Clinical features

Symptoms may be insidious and slowly progressive over weeks to months, or they may be acute and explosive. Symptoms and signs result from bone marrow failure or the involvement of extramedullary sites by leukemia (74). No infectious basis for fever is found in many patients, especially if the neutrophil count exceeds 0.2 x 10^9 /L (75). The leukemic process itself may be responsible for fever because fever resolves in most patients after the institution of chemotherapy (76). Prominent skeletal symptoms occur primarily in children with no lymphadenopathy, organomegaly, or leukocytosis. And as a result, the diagnosis of leukemia often is delayed (77, 78). Approximately 2% of children present with what appears to be aplastic anemia; overt leukemia follows within weeks to months (47, 79). Physical findings may include pallor, petechiae purpura, mucous membrane bleeding, fever, lymphadenopathy, splenomegaly, hepatomegaly, bone tenderness, and fundic hemorrhages. Abdominal organomegaly rarely is symptomatic. Skin involvement is rare; when occurs, it is associated with the pre–B–cell phenotype (80). Infants 24 months of age or younger are more likely to have massively enlarged livers and spleens, hyperleukocytosis, involvement of spinal fluid by leukemic cells, and slow response to therapy (26).

- **White blood cell count**

The total white blood cell (WBC) count at the time diagnosis is the single most powerful determinant of remission induction, remission duration, and long–term survival for all age groups (81, 82). A WBC count above 100 x 10^9/L at diagnosis is particularly devastating (83). Patients with high WBC counts often have bulky extramedullary disease at diagnosis and are at high risk for relapse in the CNS or testes after attainment of bone marrow remission. As with other prognostic factors (except for sex), the significance of the WBC count diminishes with time, so that by 24 months from diagnosis, it is no longer predictive of outcome (84).

- **Age**

The second most significant prognostic factor is age at diagnosis (74, 85). In the age group 2 to 6 years, long-term survival is more than one–and–a–half time that of children less than 2 years or more than 10 years treated in an identical manner (86). Infants less than 1 year of age have an especially poor prognosis. Adolescents in the age group 10 to 20 years are also at higher risk than younger children (87) and they may be at higher risk than adults 20 to 50 years of age (88, 89). Leukemia in adolescents, as is infants, is often associated with adverse prognostic factors including high WBC counts at diagnosis, T–cell immunophenotype, FAB L2 blast morphology, low DNA index, and ploidy other than hyperdiploidy. Despite these association, age 10 to 20 years at diagnosis of ALL has independent adverse prognostic significance (87). Adults fare less well than children, and within the adult group, age greater than 50 years is associated with less favorable remission rates. Age has a similar ordering effect on remission duration and survival. The low response rate in adults are related at least in part to age–related biologic differences of the disease. ALL in adults more often is associated with a high initial WBC count, FAB L2 morphology, the Philadelphia chromosome, and a slow response to therapy.

- **Sex**

A difference in prognosis between boys and girls did not become apparent until the introduction of CNS–directed therapy and the emergence of a population of long–term survivors. Notable is the observation that sex, unlike WBC count and age, fails to lose its prognostic significance with time (90). After discontinuation of therapy, boys continue to experience a higher incidence of relapse, which can be explained only in part by relapse in the tested, as the rate of bone marrow relapse also is higher (90). Adult men, like boys, have a less favorable survival record than adult women (81, 91). Unlike the situation in children, the difference is associated principally with a lower remission induction rate rather than a higher relapse rate.

- **Blast morphology**

Despite controversy (92), FAB morphologic classification has proved to have strong prognostic significance (82, 93). FAB L1 morphologic features are associated with more favorable prognosis than L2 in childhood ALL, whereas L3 subtype has historically been predictive of remission induction failure and short survival in both adults (81) and children (86). Because L1 and L2 morphology bear no relationship to immunology subtype or other prognostic factors (94, 95), these morphologic subtypes were predicted to have independent prognostic significance. This theory has been borne out by multivariate analysis. A higher frequency of L2 blasts is observed in adults (95).

- **Platelet count**

An association between low platelet count (less than 30 x 10^9/ L) and shorter remission duration has been noted in some studies (96). Thrombocytopenia appears to be a stronger predictor of relapse in boys and girls. Leukemic seeding associated with petechial hemorrhages may predispose patients to testicular and CNS relapse (97).

3.4.2 Laboratory features

The white blood cell (WBC) count is elevated in 60% of patients with ALL at the time of diagnosis. A WBC count in excess of 50 x 10^9/ L is often associated with prominent lymphadenopathy, hepatosplenomegaly, and the T–cell immunophenotype. Bone marrow aspiration is indicated to establish the diagnosis and obtain cells for immunologic phenotyping and histochemical and cytogenetic analysis. Bone marrow lymphoblasts are more homogenous with respect to both morphologic and biologic charateristics than those in the blood. A large leukemic cell burden having a high rate cell turnover may produce several metabolic disturbance. Chief among these is elevation of the serum uric acid level (98). Increased cell destruction also is responsible for hyperphosphatemia and hypocalcemia (99). Serum levels of lactic dehydrogenase (LDH) are increased because of an increased turnover of leukemic cells.

3.4.3 Diagnosis and differential diagnosis

The diagnosis of ALL is based on the demonstration of lymphoblasts in the bone marrow. Characterization of the leukemia as lymphoblastic or nonlymphoblastic is based on morphologic, cytochemical, immunophenotypic, cytogenetic, and molecular biologic observations.

3.4.4 Classification and assessment of risk

ALL is a heterogeneous group of disorders comprising subtypes that differ with respect to their pathogenesis, biology, and clinical features. The ability to identify biologic and clinical features that influence prognosis has led to the use of different treatment regimens for different risk groups, which in turn have resulted in both improved overall outcomes and decreased toxicities for selected subsets of patients. Current schemes for classification of risk rely primarily on clinical features Table 3.6 However, recent advances in the understanding of immunophenotypic and molecular genetic features of ALL promise to lead to an improved biologically based classification that will ultimately result in more specific biologically driven therapies.

Table 3.6 Prognostic factors in acute lymphoblastic leukemia

Determinants	**Favorable**	**Unfavorable**
Clinical features		
WBC count	<10 x 10^9/L	>50 x 10^9/L
Age	3 –7 years	< 1, > 10 years
Sex	Female	Male
Race	White	Black
Time to remission	< 14 days	> 28 days
Node, liver, spleen enlargement	Absent	Massive
Mediastinal mass	Absent	Present
CNS leukemia	Absent	Present
FAB morphologic feature	L1	L2, L3
Hemoglobin	< 7g/ dL	> 10g/ dL
Platelet	> 100 x 10^9/ L	< 30 x 10^9/ L
Serum immunoglobulin	Normal	Decreased
Immunophenotype	Early pre–B cell	T cell B cell Mixed lineages
Cytogenetic markers	Hyperdiploidy 6q-	Pseudodiploidy t(9;22) t(8;14) t(4;11) t(14q+)

WBC = white blood cell, CNS = central nervous system.

3.4.4.1 Immunophenotype

A classification of ALL based primarily on the surface marking characteristics of leukemic blast. It recongnizes two lineages of lymphocytes (T and B cells), each of which can be subclassified into several maturational stages (100, 101). Leukemic T cells differ from those of B-cell lineage in that they less often express CD10 (the common ALL antigen, or CALLA) (102). and HLA-DR, they more often are PAS negative, and they may have an increased amount of acid phosphatase (103). Mature B-cell ALL is distinguished from other B-lineage leukemia by surface immunoglobulin (sIg) expression, whereas so called pre–B express cytoplasmic, but

not surface, immunoglobulin (104). Precursor B cell, although lacking both membrane and cytoplasmic immunoglobulin express surface antigens that are shared by more mature B lyphocytes. Commitment to B cell differentiation may be identified by rearrangement of one or more immunoglobulin genes.

Early pre–B–cell ALL comprises approximately two–thirds of cases of childhood ALL (74, 105) and a somewhat lower proportion of adult cases of ALL (81, 106). In all age groups, this immunophenotype is associated with a favorable prognosis (81, 107). Although most early pre–B lymphoblasts are CALLA positive, CALLA expression does not appear to have independent prognostic significance (94, 108).

Pre–B cell ALL accounts for approximately 20% of childhood ALL (107, 109). The pre–B cell phenotype is associated with a greater risk of marrow and CNS relapse, and shorter survival than early pre-B ALL (109). Expression of cytoplasmic immunoglobulin (cIg) in ALL is associated with a non random cytogenetic abnormality, t(1;19)(q23;q13) (110). The presence of this translocation, which is identified at the cytogenetic level in 20 to 30% or pre-B ALL patients (110), is the primary determinant of adverse prognosis in pre–B ALL (77, 111) patients without this lesion have a survival rate similar to that to patients with early pre–B ALL.

Mature B cell ALL is uncommon, accounting for only 1 to 2% of ALL cases in both children and adults (94, 107). Both B–cell ALL and Burkitt lymphoma are characterized by FAB L3 morphology (33), Children with B–cell ALL are older than those with other B–lineage subtypes and have a higher incidence of CNS involvement.. Both children and adults with B–cell ALL have a poor response to, and abbreviated survival following, standard ALL therapeutic regimens (32, 112) **;** newer dose–intensive regimens used for Burkitt lymphoma has led to cure rates above 60% (113). Transitional pre–B ALL, these cases lack FAB L3 morphology or the chromosomal translocations associated with mature B–cell ALL, and have a favorable outcome.

T–cell ALL accounts for 10 to 15% of ALL in both children (24, 107) and adults (106, 114). It is rare in infants less than 1 year of age and in adults greater than 50 years of age (115). T-cell ALL occurs more often in males and is often associated with a high WBC count at diagnosis (114, 116). Although T–cell ALL was previously

associated with a poor prognosis (117), the recent use of more intensive therapeutic regimens has resulted in a survival approaching that of non T-cell ALL. The prognostic significance of T–cell ALL is related to clinical features associated with a poor prognosis, such as high initial WBC count, age greater than 15 years, massive splenomegaly, FAB L2 blast morphology, and abnormal karyotype (116). A recently identified subset of T–cell ALL characterized by expression of the T cell associated CD7 surface antigen in the absence of CD4 or CD8 is associated with resistance to conventional chemotherapy and poor survival (118, 119)**.**

Approximately 10 to 20% of children (120, 121) and 35% of adults (122) with ALL express myeloid-associated antigens such and CD13 and CD33. Although initial reports suggested that ALL with myeloid marker expression was associated with a poorer outcome (121), expression of myeloid antigens has had no adverse prognostic significance in recent clinical trials of childhood ALL (120).

Other immunophenotypic markers have prognostic significance as well. Expression of the stem cell associated surface antigen CD34 exerts an independent favorable effect on the outcome of B lineage, but not T–lineage, childhood ALL (123). Lack of expression of CD45 antigen, which is uniformly expressed in T–lineage childhood ALL but only a proportion of B–lineage ALL, was associated with lower WBC count, lower serum LDH, and hyperdiploidy in one study (4).

3.4.4.2 Cytogentic markers

Both chromosome number (ploidy) and chromosomal structural alterations have prognostic significance that is independent of other disease characteristics (4, 124).

3.4.4.2.1 Chromosome number

Patients whose blasts demonstrate a hyperdiploid karyotype (modal number of chromosomes greater than 50) experience longer remissions and better survival rates than other cytogenetic group (125, 126). The adverse prognostic significance of hyperdiploidy (47 to 50 chromosomes) seen in earlier studies has improved with the use of more effective therapy. The prognosis of children whose blasts contain 47 to 50 chromosomes is comparable to that of children with a normal

chromosome number (126), whereas adults with 47 to 50 chromosomes appear to fare less well than those with pseudodiploidy. Both children and adults with pseudodiploidy (structural rearrangement without change in total chromosome number) have short remission and poor survival (124, 126). Hypodiploidy confers a poor prognosis (127). Differences in the relative frequency of ploidy in children and adults contributes to the adverse influence of increasing age on prognosis. Approximately 43% of children have hyperdiploid clones (126), in contrast to only 12% of adults (81). Chromosome classification adds significant prognostic information to the WBC count at diagnosis, age, FAB classification, and cell immunotype (126).

3.4.4.2.2 Structural aberrations

ALL associated with the presence of genetic aberrations plays key roles in the development and function of lymphoid cells allowing the identification of prognostically relevant subgroups (92, 128).

- **TEL-AML1 rearrangement**

The t(12;21)(p13;q22), resulting in ETV6/RUNX1 gene rearrangement, fuses ETV6, which encodes an ETS family transcription factor, and RUNX1, which encodes a transcription factor with a DNA binding domain. The t(12;21)(p13;q22) was first reported by two different groups in 1995 (26). Various later studies demonstrated that this translocation, not detectable by conventional cytogenetics, constitutes the most frequent rearrangement in childhood ALL. It occurs in approximately 25% of childhood ALL (26, 80, 129). The majority of positive patients range in age between 1 and 12 years at diagnosis, with a peak between 2 and 5 years; all display a precursor-B cell immunophenotype, in particular common ALL and pre-B-ALL, more rarely pro-B-ALL. In addition, these patients are characterized by low WBC count at diagnosis (<50000/I). Interestingly, the vast majority of patients have a non-hyperdiploidy DNA content (DNA index =1), most have coexpression of myeloid markers (129) and the majority (70-80%) show deletion of the non-rearranged TEL allele (130). So far, t(12;21) has not been found in T-ALL or AML. It has never been described in infant

leukemia (age less then 1 year) and the frequency in adult leukemic patients is low (<2%) (131).

Several studies reported favorable outcomes for t(12;21)-positive patients (80, 130), in both retrospective studies and prospective studies with relatively short follow-up (132). However, analyses of patients with relapsed ALL have shown that the frequency of this translocation is similar to that at diagnosis (133). Nevertheless, the t(12;21)-positive patients retain a good prognosis and seem to have a more delayed time of relapse (133). By contrast, other groups reported a very low incidence in relapsed ALL (132, 134). This apparent discrepancy must be clarified by larger prospective studies with a long follow-up. The t(12;21) involves the TEL/ ETV6 gene on chromosome 12 and the AML1/CBFA2 gene on chromosome 21 (26). The AML1 gene is also involved in t(8;21). The TEL gene is very large and consists of eight exons, but the breakpoints cluster in a 15 kb region between exons 5 and 6 (Figure 3.6) (135). The genomic organization of the AML1 gene has not yet been completely unravelled (120), and the breakpoints can occur either in the very large intron 1 (most frequently) or in intron 2. In most cases, the TEL-AML1 fusion transcript show a joining of exon 5 (nucleotide 1033) of TEL to the second exon (nucleotide 503) of AML1. Alternative splicing causes the skipping of AML1 exon 2 (39 bp) in a minority of transcripts, resulting in two PCR bands form the same patient. Less frequently, the breakpoint occurs in AML1 intron 2, also resulting in the junction of TEL exon 5 to the third exon of AML1.

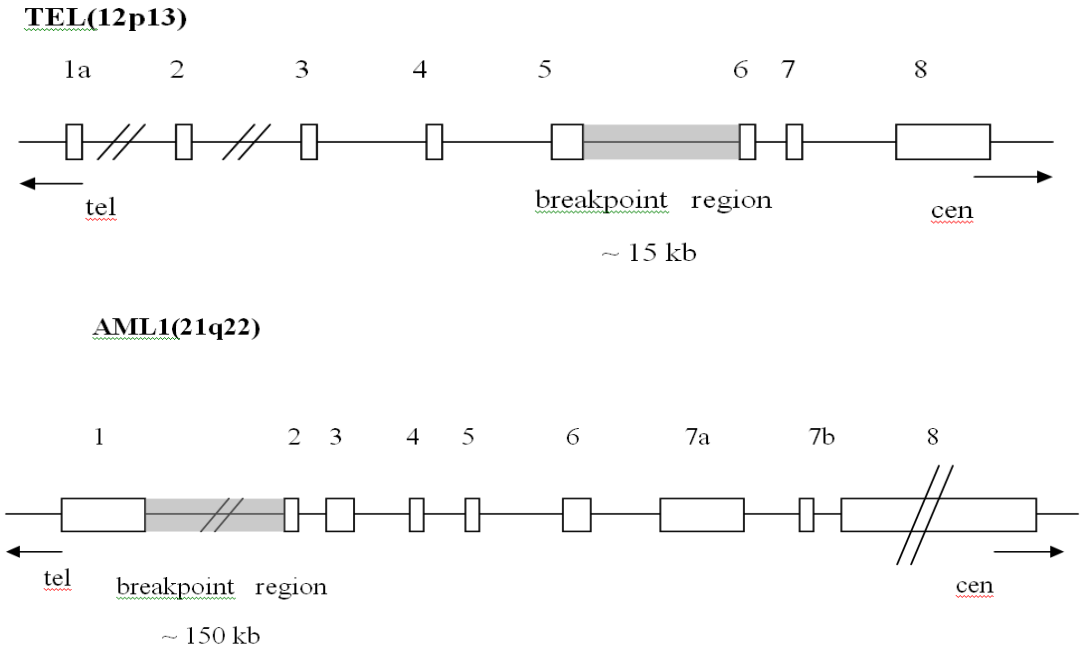

Figure 3.6 Schematic diagram of the exon/intron structure of the TEL and AML1 genes, involved in t(12;21)(p13;q22). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated.

- **BCR-ABL rearrangement**

The t(9;22)(q34;q11) occurs in 3-5% of children, the frequency increases with age and it is found in about 25% of adults with ALL. It is associated with a very poor prognosis in all age groups (136-138). Molecular analyses have established that the Ph translocation always results in the joining of 3' sequences of the tyrosine kinase c-ABL proto-oncogene on chromosome 9 to the 5' sequence of the BCR gene on chromosome 22 (139, 140). Whereas the breakpoints on chromosome 9 are generally 5' to ABL exon 2, the breakpoints on chromosome 22 differ in their position within the BCR gene, giving rise to fusion transcripts with different types of BCR-ABL junctions (140). In CML, the breakpoints on chromosome 22 are restricted to a central region of the BCR gene called 'major breakpoint cluster region' (M-bcr), leading to transcripts with different types of BCR-ABL junctions, dependent on the position of the breakpoint in BCR intron 13 or intron 14 (141). These fusion transcripts encode a BCR-ABL protein of 210 kDa, called p210 BCR/ABL. A second breakpoint cluster region in the BCR gene has been identified almost exclusively in Ph1 ALL (139). In

fact, whereas approximately 40% of Ph^1 ALL show the same molecular rearrangements as in CML, in the remaining 60% of Ph^1 ALL the BCR breakpoints are located in the so-called 'minor breakpoint cluster region' (m-bcr) between the two alternative exons and exon 2 (Figure 3.7).

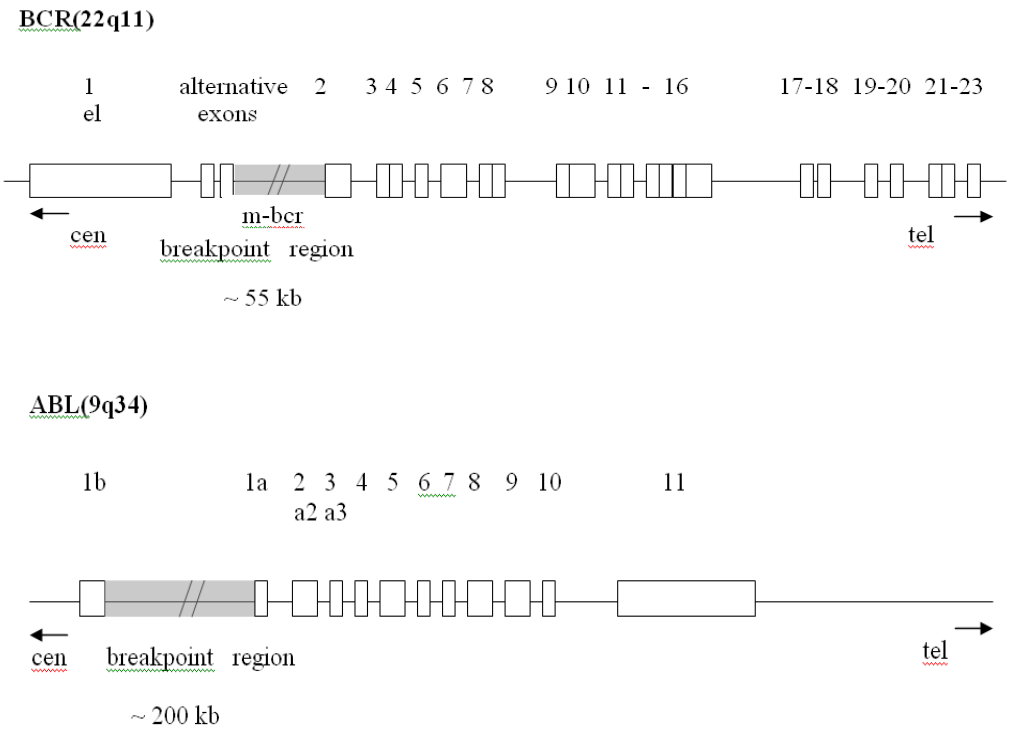

Figure 3.7 Schematic diagram of the exon/intron structure of the BCR and ABL genes, involved in t(9;22)(q34;q11) with focus on the minor breakpoint cluster region (m-bcr). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. The old nomenclature for BCR exon 1 and ABL exon 2 and 3 are also indicated.

The breakpoints in the ABL gene are virtually all located in the large intron region (~200 kb) between ABL exon 1b and exon 2 (also called exon a2). As a consequence of the m-bcr breakpoints only the first exon of the BCR gene (also called exon e1) is joined to ABL exon 2 (e1-a2 junction) (139). This results in the production of a BCR-ABL protein of 190 kDa in molecular weight (p190 BCR/ABL) (140).

Although the e1-a2 type of transcript has been mainly associated with ALL, sporadic cases of CML expressing only this type of transcript have also been reported (142). In addition, it has been found that virtually all CML patients at diagnosis, besides the usual BCR-ABL p210 transcripts, through a mechanism of alternative splicing also express low amounts of e1-a2 transcripts, whose clinical and pathogenetic significance still waits to be elucidated (143).

In ALL, the Ph^1-chromosome and consequently the BCR-ABL rearrangement are an independent unfavorable prognostic factor, which affects both hematological complete remission rate and probability of disease-free survival (144). The poor results of the conventional treatment protocols have prompted the extensive use of more aggressive consolidation approaches, which include allogeneic or autologous transplantation with either bone marrow or peripheral blood stem cells. In these treatment protocols, RT-PCR detection of BCR-ABL fusion transcripts certainly represents the method of choice for monitoring MRD during the follow-up (145). The clinical validity of this method in Ph^1 ALL has been recently demonstrated for patients receiving BMT (146). In these ALL patients the reappearance of RT-PCR positivity after BMT was significantly associated with the occurrence of a hematological relapse. Moreover, the possibility of identifying patients at high risk of relapse will eventually allow testing of the efficacy of new therapeutic strategies aiming at the decrease of post-transplant relapse rates, such as adoptive immunotherapy, antibody-based therapies, or interferon.

- **E2A-PBX1 rearrangement**

The t(1;19)(q23;p13) was first reported in 1984 by different groups in some cases of pre-B-ALL (136) and the literature data so far reported that this translocation can be found in a balanced (25%) or unbalanced (75%) form–19, +der (19)t(1;19) in which two normal chromosomes 1 are present. The t(1;19) is detected in about 5-6% of childhood ALL and in about 3% of adult ALL (77, 146). In both pediatric and adult patients this translocation occurs almost exclusively in pre-B-ALL expressing cytoplasmic Igμ, even though it has been reported sporadically in pre- B ALL and common ALL (<1%) as well as in rare cases of T-ALL and AML (77). Most cases carrying the t(1;19) express a typical immunophenotype with homogeneous expression

of CD19, CD10, CD9, complete absence of CD34, and at least partial absence of CD20 (137). Moreover, the t(1;19) correlates with the presence of known clinical high-risk features, such as elevated cell count, high serum lactate dehydrogenase levels and central nervous system involvement (77). In the majority of cases the E2A-PBX1 fusion transcript shows a constant junction site of exon 13 (nucleotide 1518) of E2A (148) to the second exon (nucleotide 388) of PBX1 (Figure 3.8). A variant fusion transcript is described in about 5-10% of t(1;19) positive ALL (149, 150). It is characterized by a stretch of 27 nucleotides inserted at the usual junction point between nucleotides 1518 and 388 of the E2A and PBX1 genes, respectively. These additional nucleotides, which are identical in each case, appear to arise form a differentially spliced exon of either E2A or PBX1 genes, but their exact derivation still remains unknown (149, 150).

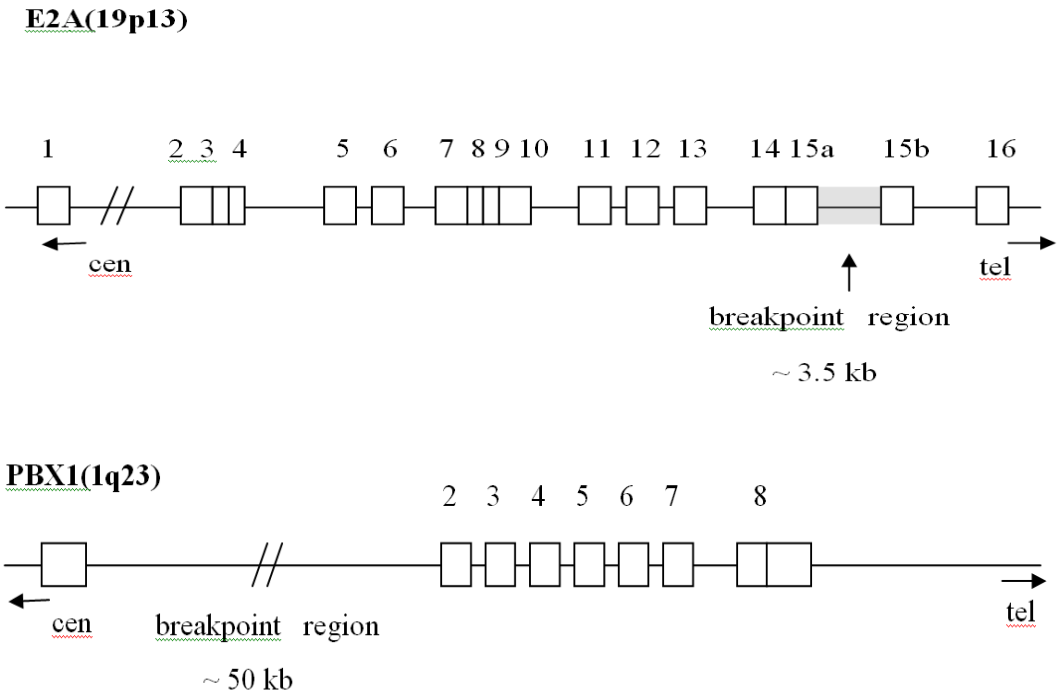

Figure 3.8 Schematic diagram of the exon/intron structure of the E2A and PBX1 genes, which are involved in t(1;19)(q23;p13).The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated.

- **MLL-AF4 rearrangement**

Molecular studies have shown that t(4;11)(q21;q23) involves the MLL (HRX, Htrx) and AF4(FEL) gene (141). MLL-AF4-positive leukemia are observed in 50-70% of infant ALL cases and in approximately 5% of pediatric and adult ALL cases (152, 153). The presence of a t(4;11)(q21;q23) has been associated with the pro-B-ALL phenotype (CyCD79a+, CD19+, CD10-, CD24-) (153) and with coexpression of myeloid differentiation antigens (CD15 and CD65) (144). The t(4;11)(q21;q23) fuses the 5' portion of MLL to the 3' portion of AF4 (Figure 3.9).

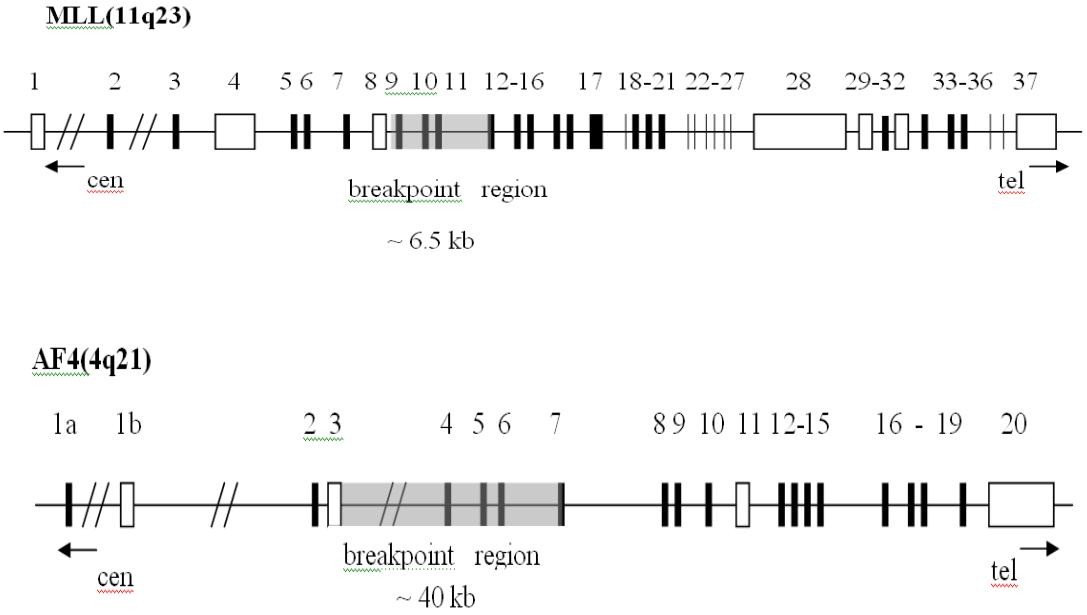

Figure 3.9 Schematic diagram of the exon/intron structure of the MLL and AF4 genes, which are involved in t(4;11)(q21;q23). The centromere (cen) and telomere (tel) orientation,exon numbering, and relevant breakpoint regions are indicated.

The MLL gene is composed of 37 exons spanning a region of > 1 MB. There are two numbering systems of exons; the most recent system will be used in this study(155). The MLL protein contains a region of three AT-hooks, which have binding capacity to the minor groove of DNA. A domain with binding capacity to hemimethylated DNA is located in the 5' portion of the protein encoded by exon 8.

A zinc finger domain is encoded by exons 11 to 16 and a drosophila trithorax homology domain is located at the 3' part of the gene. The MLL gene has been found involved in approximately 30 different translocations, of which approximately 20 with identified partner genes (156). Although some of the involved fusion partners may share sequence homology, fusion partners of completely different predicted functional properties have been characterized. MLL fusion gene are found in precursor-B-ALL, AML, myelodysplastic syndrome (MDS), some cases of T-ALL, and in secondary leukemia (157, 158).

In addition to MLL fusion genes generated by translocations, deletions of exon 11 have been associated with T-ALL (159) and tandem duplications of the gene have been observed in both *de novo* and secondary AML (160) as well as in normal peripheral blood and bone marrow mononuclear cells (161). The AF4 gene is composed of 20 exons and encodes a serine-proline-rich protein (162). The function of this protein has not been determined, and the functional domains are not well characterized. MLL-AF4 fusion genes have been found in virtually all t(4;11) and in a significant number of cases where the t(4;11) was not detected by conventional cytogenetics (144). Reciprocal AF4-MLL transcripts have been found in 70% of cases (154). In contrast to the t(8;21) or the t(12;.21), where only one or two variant fusion transcripts are observed, at least 10 different MLL-AF4 fusion transcripts have been found, due to breakpoints in different introns. Furthermore, differential splicing is a common phenomenon, leading to more than one fusion transcript in a leukemia (154).

MLL-AF4 has been identified as an adverse prognostic factor in infant leukemia by several study groups (152, 163). Also, it has been associated with a bad prognosis in adults, but prognosis seems to have improved with the introduction of high-dose Ara-C in the induction therapy of adult ALL (143). In pediatric cases, there is some suggestion that different age groups have different prognoses (152, 163). Whether there is a correlation with different types of MLL-AF4 fusion genes is still unclear.

- **Other recurrent structural abnormalities**

Other recurrent structural abnormalities in BCP-ALL include for example dic(9;20)(p13;p11), dic(9;12)(p13;p13), dic(7;9)(p11;p11), t(12;17)(p13;q12), and t(8;14)(q11.2q32) (154-156). These aberrations occur at a frequency of <2% in ALL, and their prognostic importance is not yet established.

- Chromosomal translocations involving the immunoglobulin heavy-chain locus (IGH) are a feature of mature B-cell malignancies and result in deregulated expression of the translocated genes due to the proximity of transcriptional enhancers within IGH. The partner genes vary according to the disease subtype (167).

- PAX5 encodes the only PAX protein expressed within the haematopoietic system, the B-cell lineage specific activator protein (BSAP) that is required for B-cell commitment and maintenance. At the molecular level, Pax5 fulfils a dual role by activating B-cell specific genes and simultaneously repressing lineage-inappropriate genes (168). In the bone marrow, Pax5 is exclusively expressed from the pro-B to the mature B-cell stage and is downregulated during terminal differentiation into plasma cells (169).

- **Minimal residual disease**

The ability to detect leukemic clone–specific genetic alterations in bone marrow samples of patients with ALL in clinical remission by the highly sensitive polymerase chain reaction (170, 171), or by other methods such as flow cytometry combined with leukemic progenitor–cell assays (172), has generated enthusiasm for monitoring of so–called minimal residual disease, with the promise of further tailor therapy based on identification of persistent disease after clinical remission. A number of recent studies have demonstrated the ability to identify impending relapse in subsets of patients, based on correlation of detection of clonal abnormalities with subsequent relapse (170, 171). However, wide variation in the sensitivity and specificity of the methods used to detect minimal residual disease, as well as the unexpected finding of persistent genetic abnormalities related to the leukemic clone in patients in apparent sustained remissions (171) have not yet established a role for these studies in patient management (173).

3.4.5. Prognostic stratification

Because treatment is the single most important prognostic factor, the relative prognostic power of disease characteristics varies form study to study. Consequently, different sets of prognostic variables have been found useful. These include the WBC count, age, race, and karyotype ploidy (128), WBC count and size of liver and spleen (92), and WBC count and age alone (46). The Children's Cancer Study Group used the latter two variables to identify three prognostic groups within a large population of patients less than 21 years of age treated in a uniform fashion. Children between 3 and 7 years of age with an initial WBC count less than 30 x 10^9/ L had a 4–year continuous remission rate of nearly 90%. This good prognosis group accounted for 27% of the study population. Average–risk patients were defined as those of all ages with an initial WBC count between 10 and 50 x 10^9/L and those younger than 3 years or older than 7 years with a WBC count less than 10 x 10^9/ L. This group constituted 54% of the total and had a 4-year continuous remission rate of approximately 60%. High–risk patients, identified by a WBC count above 50 x 10^9/ L, made up 19% of the population and had a median survival of only 2 years. This stratification was used prospectively to evaluate different therapeutic strategies for different prognostic group (46). The Children's Cancer Study Group subsequently used the WBC count at diagnosis, age, sex, extent of extramedullary disease, FAB morphologic classification, and platelet count to stratify patients into five groups that differ with respect to prognosis, relapse patterns, and therapeutic priorities (75). Difference among the risk classification criteria used in clinical trials of childhood ALL has heretofore rendered difficult, if not impossible, accurate comparisons of outcomes that ate the consequence of varying treatment strategies across organizations. To overcome this obstacle, a consensus workshop sponsored by the NCI in collaboration with representatives form major organizations involved in the design and conduct of therapeutic trials for childhood ALL led to the development of uniform criteria for risk–based treatment assignment. Henceforth, the standard–risk group of patients will include those with B-precursor ALL ages 1 to 9 years with a WBC count less than 50,000/µl, with an estimated event–free survival (EFS) of approximately 80%: the remaining high–risk patients have an estimated EFS of approximately 65%. As different treatment strategies have yielded varying conclusions

regarding the prognostic significance of the T–cell phenotype, some group classify T–cell patients according to WBC count and age, whereas others classify all T–cell ALL patients as high risk. Other prognostic factors that will be obtained in all patients include DNA index, cytogenetics, early response to treatment, immunophenotype, and CNS status.

The successful intensification of therapy for high–risk ALL has weakened the power of many adverse predictive factors. Many examples are now available of the potential of newer treatment strategies to offset the negative impact of clinical and biologic variables, including high WBC counts and splenomegaly (76, 92), the T-cell immunophenotype (120, 174), myeloid–associated antigen expression by ALL blast (65), t(1;19) in pre–B cell ALL (76), pseudodiploidy, and hypodiploidy (175).

CHAPTER IV
MATERIALS AND METHODS

4.1 Biological samples

Patient : The total of 32 bone marrow samples were collected from ALL patients from Faculty of Medicine Ramathibodi Hospital, Faculty of Medicine Chulalongkorn University, Nakornpathom Hospital, Phrapinklao Hospital and Faculty of Medicine King Phramongkutklao, Thailand during September 2008 to September 2009. All have been previously diagnosed as acute leukemia by using clinical manifestation, morphological, cytochemical, and immunophenotyping methods. The document for sample collection was submitted to Ramathibodi Hospital Ethic Committee.

Normal individuals: Peripheral blood from 5 normal individuals without evidence of hematologic malignancies or solid tumor for 5 years later were performed as cut-off point for I-FISH. technique.

4.2 Material

4.2.1 Instruments and laboratory supplies for FISH

- 0.2 µm pore filtration unit
- Autopipette (0.5-2 µl, 10 µl, 100 µl, 200 µl and 1000 µl)
- Centrifuge for conical 15 ml tube
  (Eppendorf AG · 22331 Hamburg Germany)
- Conical 15 ml tube
- Coverslips (18mm X 18mm)
- Olympus fluorescence with image analyzer

(applied imaging Olympus optical Co.,Ltd, Tokyo, Japan)

- Forceps
- Glass bottle Duran (50cc, 500cc and 1000cc)
- Glass Coplin jar
- Graduated cylinder 1000 mL
- Incubator (37 ^{o}C)
- Microcentrifuge tube
- Moisture box
- Pasture pipette 1 box
- pH meter
- Refrigerator
- Spin down
- Thermobrite (Vysis, USA)
- Thermometer (range 37-100^{o}C)
- Timer Magnetic stirrer
- Tip (yellow, blue, white)
- Vortex mixer
- Water bath (37 ^{o}C and 73^{o}C)

4.2.2 Reagent for FISH

4.2.2.1 Reagent for cell preparation

- Hypotonic solution (0.56% KCl)
- 25XPBS
- Standard methanol : acetic acid (3:1) fixation

4.2.2.2 Reagent for hybridization

- 70% ethanol (Merck, Germany)
- 85% ethanol (Merck, Germany)
- 100% ethanol (Merck, Germany)
- 20XSSC
- 2XSSC
- Denaturation Solution (70% Formamide/ 2XSSC)
- RNase A
- Pepsin A
- 70% Formaldehyde
- 1N HCl
- 1M $MgCl_2$

4.2.2.3 Reagent for washing and detection

- 0.4XSSC/ 0.3%NP-40 Wash solution
- 2XSSC/ 0.1%NP-40 Wash solution
- DAPI solution (Vysis, USA)

4.2.2.4 Reagent for probe preparation

- Hybridization buffer (Vysis, USA)

4.2.3 The DNA probes

4.2.3.1 DNA probe was purchased from Vysis, U.S.A as followed:

a. The LSI TEL/ AML1 ES Dual Color, Translocation Probe

The LSI TEL/ AML1 ES Dual Color Translocation Probe is a mixture of the LSI TEL probe labeled with SpectrumGreen and the LSI AML1 probe labeled with SpectrumOrange. The LSI TEL probe begins between exons 3-5 and extends approximately 350 kb toward the telomere on chromosome 12. The approximately 500 kb AML1 probe spans the entire gene. In a normal nucleus, the expected pattern for a cell hybridized with the LSI TEL/ AML1 ES Dual Color Translocation probe is the two orange (AML1), two green (TEL) (2O2G) signal pattern. The expected signal pattern from cells with a positive result contain two orange (AML1), one green (TEL) and fused orange and green signals which may sometime be percieved as yellow (2O1G1F) signal pattern

b. The LSI ETV6 Dual Color, Break Apart Rearrangement Probe

The LSI ETV6 Dual Color, Break Apart Rearrangement Probe targets rearrangements at the ETV6 gene region, and is a mixture of two probes. The first probe, a 630 kb probe labeled in SpectrumGreen begins about 6 kb proximal to the ETV6 (TEL) gene and extends to toward the centromere. The second probe, labeled in SpectrumOrange, begins within ETV6 intron 2 and extends toward the 12p telomere for approximately 490 kb. There is a gap between the two probes of about 140 kb. Hybridization of this probe to interphase nuclei of normal cells is expected to produce two pair of overlapping, or nearly overlapping, orange and green (yellow fusion) signals (2F) The anticipated signal pattern in abnormal cells having a chromosomal breakpoint within the gap between the two probe targets on one chromosome 12 is one orange, one green, and one fusion signal (1O1G1F).

c. The LSI TCF3/ PBX1 Dual Color, Dual Fusion Translocation Probe

The LSI TCF3/ PBX1 Dual Color, Dual Fusion Translocation Probe, the TCF3.SpectrumGreen probe is 730 kb in size and extends beyond the TCF3 gene to cover a larger region on chromosome 19p13.3. The PBX1 SpectrumOrange probe is

635 kb in size and covers the entire PBX1 gene on chromosome 1q23. Pattern of normal and abnormal nuclei are the same TEL/ AML1 probe (2O2G and 2O1G1F respectively).

d. The LSI BCR/ ABL Dual Color, Dual Fusion Translocation Probe

The LSI BCR/ ABL Dual Color, Dual Fusion Translocation Probe is a mixture of the LSI BCR probe labeled with SpectrumGreen and the LSI ABL probe labeled with SpectrumOrange. The spanning ABL probe has a genomic target of approximately 650 kb extending from an area centromeric of the argininosuccinate synthetase gene (ASS) to well telomeric of the last ABL exon. The BCR probe target spans a genomic distance of about 1.5 Mb. The BCR probe begins within the variable segments of the immunoglobulin lambda light chain locus (IGLV), extends along chromosome 22 through the BCR gene, and ends at a point approximately 900 kb telomeric of BCR. A region of about 300 kb containing low-copy number repeats has been eliminated from the probe which introduces a gap in the coverage of the probe target. Both probes span their respective breakpoints. A normal nucleus will exhibit two orange, two green (2O2G) signal pattern. An abnormal nucleus. One orange, one green and two orange/ green fusion signals are observed (1O1G2F).

e. The LSI MLL Dual Color, Break Apart Rearrangement Probe

The LSI MLL Dual Color, Break Apart Rearrangement Probe consists of a 350 kb portion centromeric of the MLL gene breakpoint cluster region (bcr) labeled in SpectrumGreen and approximately 190 kb portion largely telomeric of the bcr labeled in SpectrumOrange. The signal pattern observed in a cell lacking the MLL rearrangement is expected to show a two orange/ green (yellow) fusion signal pattern (2F). In a cell possessing a MLL translocation, the expected pattern is one green/orange (yellow) fusion signal, one orange signal, and one green (1O1G1F) signal.

f. The LSI IGH Dual Color, Break Apart Rearrangement Probe

The LSI IGH Dual Color, Break Apart Rearrangement Probe is a mixture of two probes that hybridize to opposite sides of the J through constant

IGH locus. The approximately 900 kb SpectrumGreen labeled LSI IGHV probe covers essentially the entire IGH variable region. The hybridization target of the approximately 250 kb SpectrumOrange labeled LSI IGH 3' flanking probe lies completely 3' to the IGH locus. As a result of this probe design, any translocation with a breakpoint at the J segments or within switch sequences should produce separate orange and green signals. When hybridized to a normal nucleus, the LSI IGH Dual Color, Break Apart Rearrangement Probe produces a two orange/green (yellow) fusion (2F) signal pattern. As there is no probe targeted to the J or constant regions, a slight gap between the two differently colored probe signals may sometimes be observed in nuclei from normal cells. When the IGH Dual Color, Break Apart Translocation Probe is hybridized to a nucleus containing an IGH translocation, one orange, one green, and one orange/green fusion signal pattern is observed (1O1G1F).

4.2.3.2 DNA probe was purchased from DakoCytomation, Denmark as follow

a. TCF3 FISH DNA Probe, Split Signal

TCF3 FISH DNA Probe, Split Signal, the Texas Red-labelled DNA-probe (TCF3-Upstream) anneals to a 286 kb area centromeric to the TCF3 breakpoint cluster region on chromosome 19p13. The fluorescein-labelled DNA-probe (TCF3-Downstream) anneals to a 567 kb area telomeric to the TCF3 breakpoint cluster region on chromosome 19p13. Co-localization of the probes results in a yellow signal(2F), whereas translocation events in the region will split the signal in separate green (fluorescein) and red (Texas Red) signals (1O1G1F).

b. PAX5 FISH DNA Probe, Split Signal

PAX5 FISH DNA Probe, Split Signal, is recommended for the detection of translocations involving the PAX5 locus at chromosome 9p13. The Texas Red-labelled DNA probe (PAX5-TR) binds to a 354 kb segment centromeric to the PAX5 breakpoint cluster region on chromosome 9p13. The fluorescein-labelled DNA probe (PAX5-Flu) binds to a 621 kb segment telomeric to the PAX5 breakpoint cluster region on chromosome 9p13. Co-localization of the probes results in a red/green

signal (2F), whereas translocation events in the breakpoint cluster region will split one signal into separate green (fluorescein) and red (Texas Red) signals (1O1G1F).

4.3 Method

4.3.1 Preparation of interphase cells (176)

4.3.1.1 Harvest cell

The EDTA bone marrow was centrifuged at 1,300 rpm for 7 minutes. The buffy coat was removed into a new centrifuge tube and washed by 1XPBS about 10 ml before centrifuged at 1300 rpm for 7 minutes. The supernatant was aspirated and resuspended the pallet. The cell pellet was subsequently washed by 0.56% KCl about 7 ml for two times. Then the fixative reagent was added slowly about 5 ml down the side of tube during mixing cell pellet with fixative. The suspension was centrifuged at 1,300 rpm for 7 minutes. The fixative step was repeated twice.

4.3.1.2 Fixed cell

One drop of cell suspension was applied on slide and allowed to air dry before hybridization. If the slide could not be prepared within 7 days, cell pallet could be stored at -20^{0}C for a long time prior to use.The cell suspension was washed twice before used as described above.

4.3.1.3 Slide pretreatment

After thawing, slide was air dry at room temperature. The slide was soked twice in 2XSSC at 37^{0}C for 1 hour. Then the slide was treated by RNase at 37^{0}C for 30 minutes to remove any endogenous hybridizable RNA species. After RNase digestion, the slide was washed in two changes of 2XSSC at 37^{0}C for 5 minutes each, to remove excess RNase, then transferred into 50 µg pepsin 0.1 N HCl (prewarmed at 37^{0}C) and incubated for 10 min. The slide was washed in 1XPBS at 25^{0}C for 5 minutes. Then the slide was fixed in 1% formaldehyde fixation solution at 25^{0}C for 12 minutes. Afterthat, the slide was washed with 1XPBS at 25^{0}C for 5 min then dehydrated in 70%, 85%,100% ethanol series and air dried.

4.3.2 Fluorescence *in situ* hybridization (FISH)

FISH analysis was performed on interphase cells using multiple commercially available probes (Vysis, USA and DakoCytomation, Denmark). These include the LSI TEL/ AML1 ES dual color translocation for t(12;21), the LSI ETV6(TEL) dual color, break apart rearrangement for 12p13,the LSI TCF3/ PBX1 dual color, dual fusion translocation for t(1;19)(q23;p13.3), the TCF3 FISH DNA Probe, Split Signal for 19p13, the LSI BCR/ ABL dual color, dual fusion translocation for t(9;22)(q34;q11.2), the LSI MLL dual color, break apart rearrangement for 11q23, the LSI IGH dual color, break apart rearrangement for 14q32, and PAX5 FISH DNA Probe, Split Signal for9p13.

Although FISH techniques has been followed by the manufacturers' instructions (Abbott Diagnostics, USA and DakoCytomation, Denmark), we present optimized condition of this technique as followed.

4.3.2.1 Probe preparation

7 µl LSI hybridization buffer, 1 µl probe and 2 µl purified H_2O was added to microcentrifuge tube at ambient temperature and centrifuged at 1,300 rpm for 1-3 seconds. The solution was vortexed and centrifugd again. Then the tube was placed in 73^0C waterbath for 5 minutes. Optimized the concentration of probe used was reduced from 1µl, to 0.5 and 0.3 µl. In this study, the suitable condition for probe preparation was 0.3 µl of probe, 2.3 µl of buffer and 1.3 µl of distilled water.

4.3.2.2 Hybridization

The slide was denatured in denaturizing solution (70% formamide/ 2XSSC) by immersed the slide in thermobrite at 73 $\pm$ 1^0C for 5 minutes**.** The slide was dehydrated in cold ethanol series and put on slide warmer about 2 min. The DNA probe was applied on target area. The slide was covered with coverslip and sealed with rubbercement. The hybridization was took place at 37^0C for 4 hours to overnight in thermobrite. The incubation period should be approximately 16-24 hours, for the good result. However, it was found from the studies that if incubation time were longer than 24 hours, high background would be observed.

4.3.2.3 Detection of probe

After hybridization, the coverslip was removed and immediately placed into wash bath contained 0.4XSSC/ 0.3%NP-40 wash solution at 73^0C for 5 minutes. The slide was washed by 2XSSC/ 0.1%NP-40 for 3 minutes at 25^0C then counter stained with DAPI and mounted with anti-fading solution. The slide was determined under fluorescence microscope connect with computerized image analysis system(applied imaging, Olympus optical Co.,Ltd, Tokyo, Japan). The filter set was appropriate for fluorophored labeled on the probe was used. The relationship between the translocation and transformation will be applied to detect and define the fusion transcripts related to the specific lymphoid malignancies.

CHAPTER V
RESULTS

5.1 The clinical data of ALL patient

Basic clinical data and laboratory result of the 32 ALL patients were summarized in Table 5.1 These included patients's age, sex, WBC at diagnosis, and cell lineage.

5.2 The cut -off point of healty volunteer

The cut-off point were performed on direct preparation of five healthy volunteers. Constellations of I-FISH was investigated in minimum of 200 cell from each case. Each cut-off point was set at mean proportion of cells with rearrangements plus 3 x standard deviations and considered as the minimum cut-off point. The cut-off point were as follows: 2.5% for TEL/ AML1 fusion signal; 1.7% for MLL split signal; 18% for IGH split signal, 4% for TCF3 split signal, 2.5% for PAX5 split signal and 0% for the ETV6(TEL) split signal; BCR/ ABL fusion signal; and TCF3/ PBX1 (Table 5.2.)

The cut -off point was different in each laboratory.

5.3 Result of I-FISH technique in ALL patients

Normal signal pattern of I-FISH technique in ALL patients was found in the percentage of 15.6% (5/32). The percentage of abnormal signal pattern detected by FISH in each case of 32 ALL patients was showed in table 5.3 and Figure 5.1-5.8. These abnormalities were translocation, split signal, multiple copies of gene, and gene deletion (not included case no.26). The percentage of translocation gene was found in 31.2% (10/32) for TEL/ AML1, 3.1% (1/32) for TCF3/ PBX1, 6.2 % (2/32) for BCR/ ABL. Split signal was observed in the percentage of 31.2% for ETV6 (TEL), 3.1% (1/32) for TCF3, 3.1% (1/32) for MLL, 9.4% (3/32) for IGH, and 3.1% (1/32) for PAX5 gene. Infrequent or new aberrations such as: multiple copies of AML1,

ETV6(TEL), PBX1, BCR, ABL, IGH, and PAX5 gene; deletion of ETV6(TEL), TCF3, IGH, and PAX5 gene were also observed. Frequency of these abnormalities was shown in Figure 5.9. In case no.26, the pattern of all probes that are available were mosaic, it diificult to interprete. The signal pattern in several probe was showed: some cell had amplification of gene (Figure 5.10), multiple copies gene, and mixed types of abnormalities.

5.4 The qualitative correlation in the result of karyotype and FISH

The qualitative correlation in the result of karyotype and FISH was found in 14 from 25 ALL patients. However the uncorrelated result was showed in 10 of 11 samples of ALL patients revealing normal karyotypic pattern with contrasted to abnormal result by FISH technique and the other 1 samples with abnormal karyotype pattern but normal by FISH technique (Table 5.4).

A positive result was found in 56.2% (18/36) of patients tested with the TEL/ AML1 probe. In the subset, 31.2% (10/32) were TEL/ AML1 fusion. The fusion was observed with multiple copies of AML1 gene (case no.22), deletion of TEL gene (case no. 5, 12, 15, and 24), and combination with other gene (case no.16 and 31). The other abnormalities without TEL/ AML1 fusion were found in 25% (8/32), gain of AML1 gene (case no.10) or with other gene (case no.1, 2, 6, 7, 8, 32, and 21,) were observed. About ETV6(TEL) split signal probe was correlated with TEL/ AML1 probe in the same case and we used this probe for detected unkown multiple partner gene that translocated with ETV6(TEL) gene. The majority of cases with TEL/ AML1 fusion were normal and the gains of gene were in agreement with the karyotype.

The TCF3/ PBX1 positive cases was found 9.4% (3/32). Only one case had TCF3/ PBX1 translocation with deletion of TCF3 gene (case no.18), this case had no result of karyotype. The other had not found translocation but gain of PBX1 gene and these cases coexistence with other gene (case no.6 and 8).

The BCR/ ABL positive cases was found 12.5% (4/32): one case had BCR/ ABL translocation (case no.13) and data correlated with karyotype, one case had BCR/ ABL translocation and combination with other gene (case no.6), the other not

found translocation but gain of ABL gene and combination with other gene (case no.17 and 32).

The MLL positive case was found 3.1% (1/32). This case showed split signal and data correlated with karyotype (case no.29).

IGH positive case was found 28.1% (9/32): one case had split signal (case no.3) and correlated with karyotype, two case had split signal and coexistence with other gene, and the other showed without split signal but observed that gain of IGH gene and coexistence with other gene (case no.1, 2, 7, 8, 17, 32).

PAX5 positive case was found 21.9% (7/32). One had split signal but no data not showed in karyotype (case no.20). The other was not found split signal: one case had deletion of PAX5 gene but normal karyotype, the other showed deletion or gain of PAX5 gene and coexistence with other gene (case no.6, 17, 31,32).

The coexistence gene were found 31.2% (10/32). Seven patients without translocation: two had multiple copies of TEL gene and IGH gene (case no.1, 2), one had multiple copies of TEL gene, AML1 gene, BCR gene, and IGH gene (case no.3), one had deletion of TEL gene combination with multiple copies of AML1 gene, PBX1 gene, and IGH gene (case no.8), two had gain of AML1 gene combination with deletion of PAX5 gene deletion (case no.9, 11), two had multiple copies of AML1 gene, ABL gene, IGH gene, and PAX5 gene (case no.17, 32). Three with translocation: one had BCR/ ABL fusion gene combination with IGH gene split signal, deletion of IGH gene, multiple copies of PBX1 gene, and deletion of PAX5 gene (case no.6), one had TEL/AML1 fusion gene with multiple copies of AML1 gene and deletion of TEL gene combination with IGH gene split signal and IGH gene deletion (case no.21), the other had TEL/AML1 fusion gene with TEL gene deletion combination with deletion of PAX5 gene (case no.31).

Table 5.1 Clinical data and laboratory result

Case no.	Sex/Age	WBC at diagnosis (/ µL)	Cell lineage
1	M/10	88,400	Early precursor B ALL
2	F/11	5,900	Precursor B ALL (relapse)
3	M/11	16,050	B ALL (L3)
4	F/3.11	4,690	Precursor B ALL
5	F/2.9	5,070	Early precursor B ALL
6	F/9.4	6,420	Early precursor B ALL (relapse)
7	M/1.1	16,550	Early precursor B ALL
8	M/7	8,000	Precursor B ALL (relapse)
9	M/5	180,000	Early precursor B ALL (relapse)
10	M/2.2	2,100	Precursor B ALL
11	M/4.8	24,820	Early precursor B ALL
12	M/2	13,640	Precursor B ALL
13	M/12	408,200	Early precursor B ALL
14	M/10.1	2,600	Early precursor B ALL
15	M/5	2,070	Early precursor B ALL
16	M/12.5	6,630	Early precursor B ALL
17	M/2.5	5,050	Early precursor B ALL
18	F/4.6	219,600	B lineage ALL
19	M/4.5	38,700	Early precursor B ALL
20	F/2.9	9,100	Precursor B ALL
21	M/4.9	51,630	Early precursor B ALL
22	M/3.4	5,800	Early precursor B ALL
23	F/6.8	5,260	Early precursor B ALL
24	F/4	8,680	Precursor B ALL
25	F/7	88,130	B lineage ALL
26	M/2	74,100	Early precursor B ALL
27	F/13	28,770	Precursor B ALL
28	F/3.8	14,200	B lineage ALL
29	F/0.11	147,300	Early precursor B ALL
30	F/5.2	14,900	Early precursor B ALL
31	M/3	4,950	Precursor B ALL
32	M/2	3,690	Early precursor B ALL

Table 5.2 The cut- off point of five healty volunteers

No	TEL/AML1		ETV6(TEL)		TCF3/PBX1		TCF3		BCR/ABL		IGH		MLL		PAX5	
	N	**Ab**	**N**	**Ab**	**N**	**Ab**	**N**	**Ab**	**N**	**Ab**	**N**	**Ab**	**N**	**Ab**	**N**	**Ab**
1	198	2	200	0	200	0	196	4	200	0	190	10	200	0	200	0
2	200	0	200	0	200	0	196	4	200	0	188	12	200	0	200	0
3	200	0	200	0	200	0	200	0	200	0	188	12	197	3	200	0
4	200	0	200	0	200	0	200	0	200	0	192	8	200	0	200	0
5	200	0	200	0	200	0	200	0	200	0	186	14	200	0	198	2
Mean(Ab)	0.4		0		0		1.6		0		11.2		0.6		0.4	
SD(Ab)	0.7		0		0		0.8		0		2.3		0.4		0.7	
Mean+3SD	2.5		0		0		4		0		18		1.7		2.5	

N = Normal, Ab = Abnormal

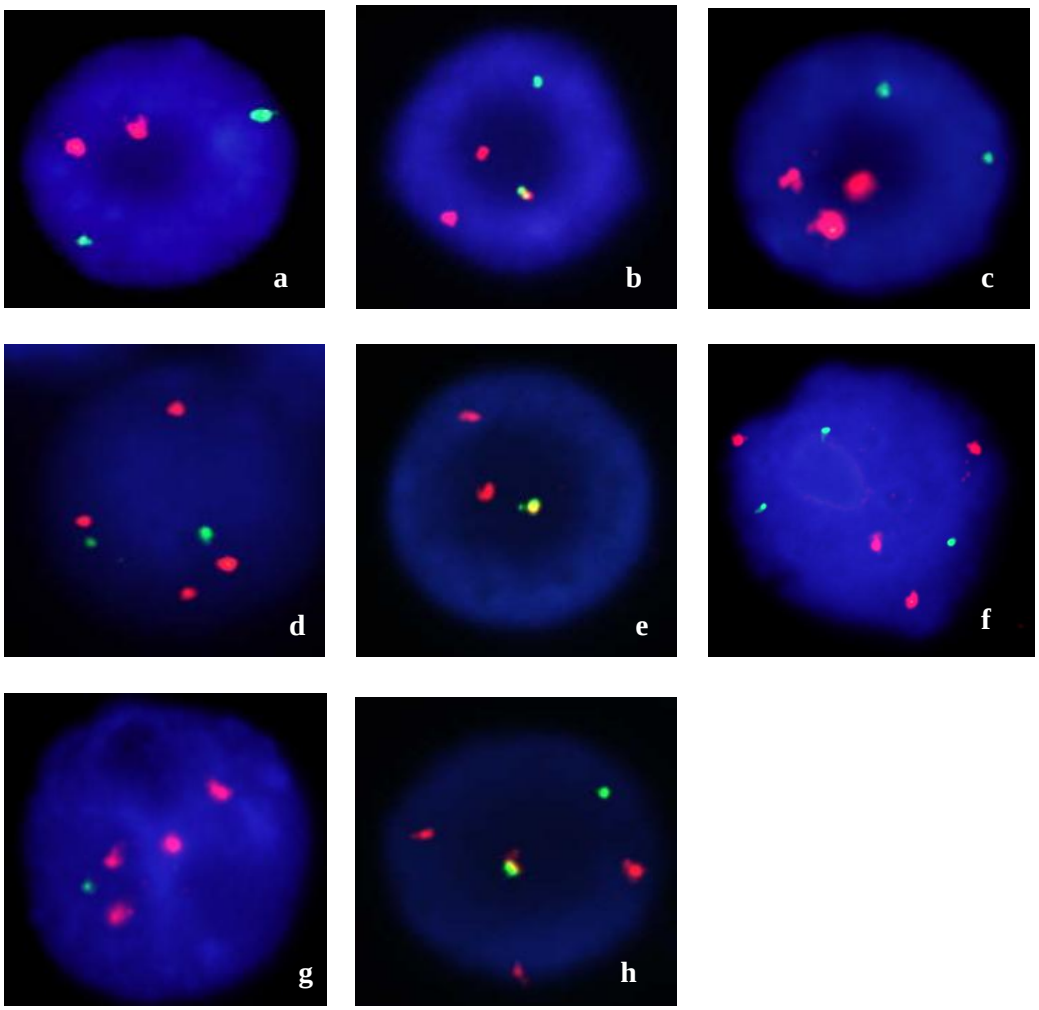

Figure 5.1 Fusion-signal FISH for detection of TEL/ AML1 gene

a: An interphase cell with two orange signals (AML1) and two green signals (TEL) are normal, no evidence of abnormality (2O2G).

b: An interphase cell with two orange signals, one green signal , and one fusion signal are typical pattern for TEL/ AML1 fusion gene (2O1G1F).

c: An interphase cell with three orange signals and two green signals are three copies AML1, not found translocation (3O2G).

d: An interphase cell with four orange signals and two green signals are four copies of AML1 gene, not found translocation (4O2G).

e: An interphase cell with two orange signals and one fusion signal are TEL/ AML1 fusion with deletion of a TEL gene (missing a green signal from the cell) (2O1F).

f: An interphase cell with four orange signals and three green signals are four copies of AML1 gene and three copies of TEL gene, not found translocation (4O3G).
g: An interphase cell with four orange signals and one green signal are four copies of AML1 gene with deletion of a TEL gene (missing a green signal from the cell) , not found translocation (4O1G).
h: An interphase cell with three orange signals, one green signal , and one fusion signal are TEL/ AML1 fusion gene with gain of a AML1 gene (3O1G1F).

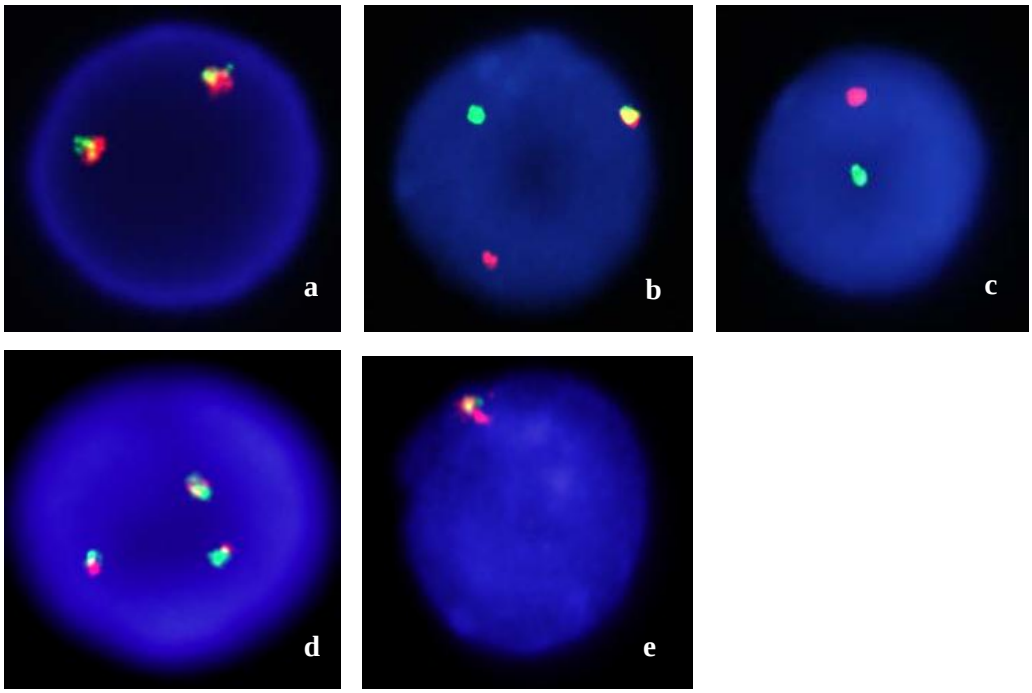

Figure 5.2 Split-signal FISH for detection of breaks in the ETV6(TEL) gene
a: An interphase cell with two fusion signals (ETV6) are normal, no evidence of abnormality(2F).
b: An interphase cell with one orange signal , one green signal , and one fusion signal are split signal of ETV6 gene (1O1G1F).
c: An interphase cell with one orange signal and one green signal are split signal of ETV6 gene with deletion of ETV6 gene (missing a fusion signal from the cell)(1O1G).
d: An interphase cell with three fusion signals are three copies of ETV6 gene (3F).
e: An interphase cell with only one fusion signal are ETV6 gene deletion (missing a fusion signal from the cell) (1F).

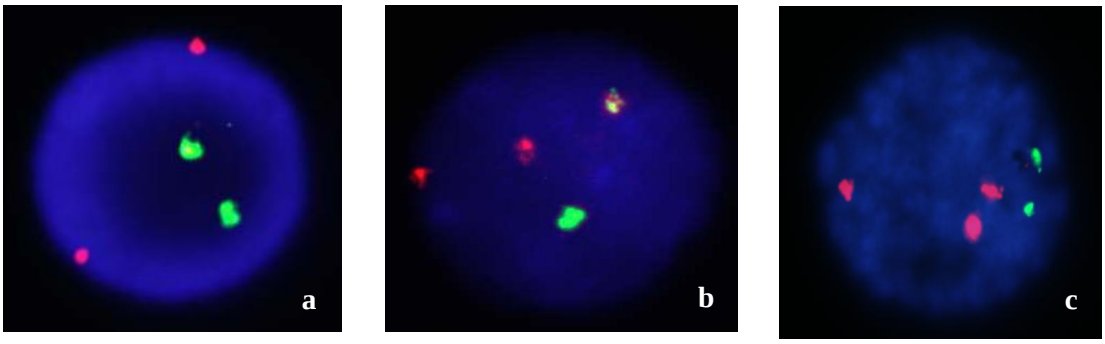

Figure 5.3 Fusion-signal FISH for detection of TCF3/ PBX1 gene

a: An interphase cell with two orange signals (PBX1) and two green signals (TCF3) are normal, no evidence of abnormality (2O2G).

b: An interphase cell with two orange signals, one green signal, and one fusion signal are typical pattern for TCF3/ PBX1 fusion gene (2O1G1F).

c: An interphase cell with three orange signals (PBX1) and two green signals (TCF3), not found translocation (3O2G).

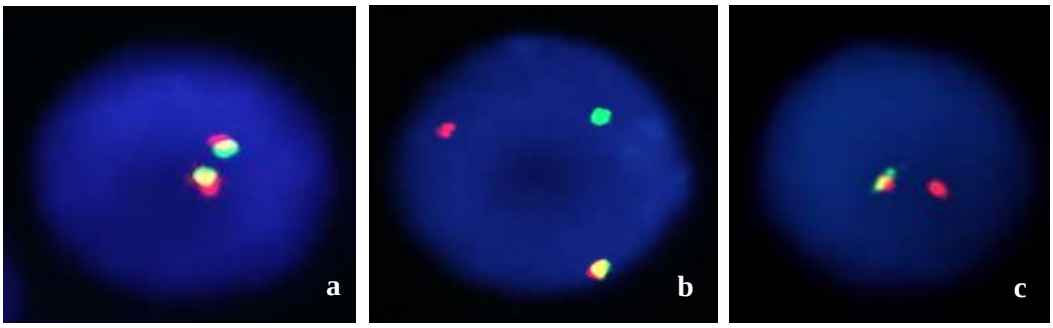

Figure 5.4 Split-signal FISH for detection of breaks in theTCF3 gene

a: An interphase cell with two fusion signals (TCF3) are normal, no evidence of abnormality(2F).

b: An interphase cell with one orange signal, one green signal, and one fusion signal are split signal of TCF3 gene (1O1G1F).

c: An interphase cell with one orange signal and one fusion signal are split signal of TCF3 gene with telomeric segment deletion of TCF3 gene (missing a green signal from the cell) (1O1F).

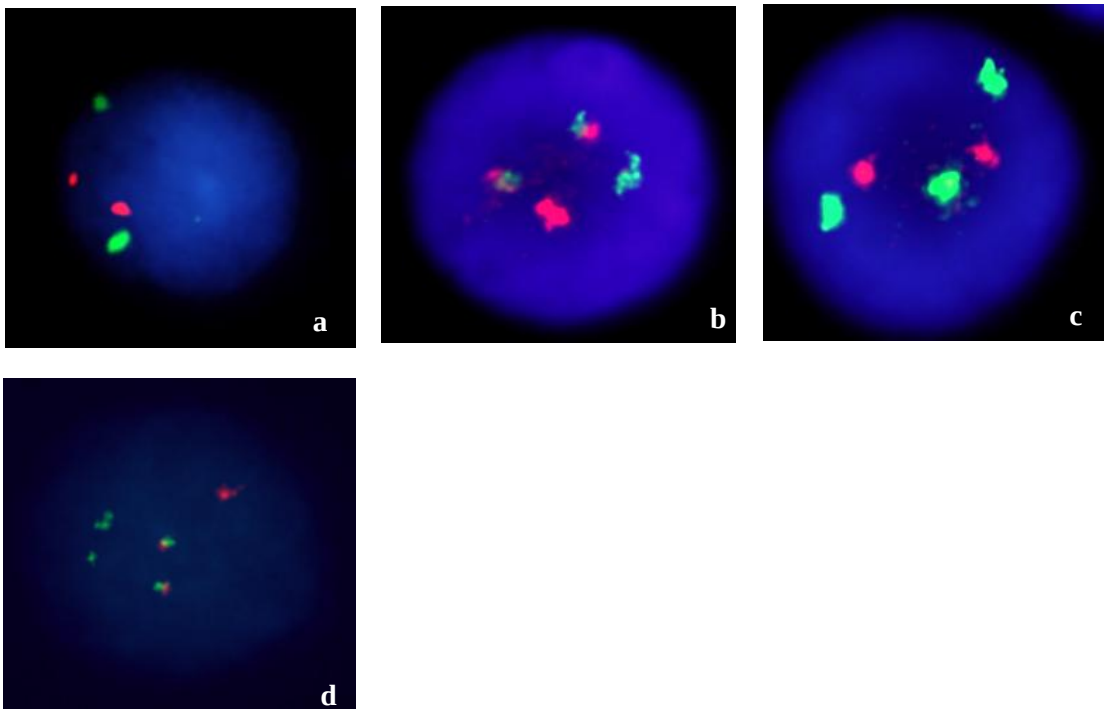

Figure 5.5 Fusion-signal FISH for detection of BCR/ ABL gene

a: An interphase cell with two orange signals (ABL) and two green signals (BCR) are normal, no evidence of abnormality (2O2G).

b: An interphase cell with two orange signals, one green signal , and one fusion signal are typical pattern for BCR/ ABL fusion gene (1O1G2F).

c: An interphase cell three green signals are three copies of BCR gene (2O3G).

d: An interphase cell with one orange signal, two green signals , and two fusion signals are BCR/ ABL fusion gene and gain of a ABL gene (1O2G2F).

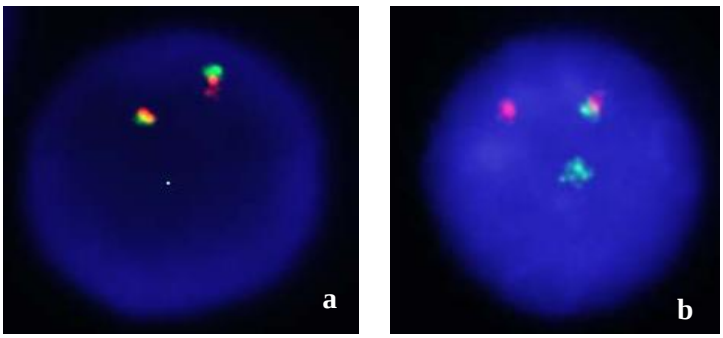

Figure 5.6 Split-signal FISH for detection of breaks in the MLL gene

a: An interphase cell with two fusion signal (MLL) are normal (2F).

b: An interphase cell with one orange, one green, and one fusion signal are split signal of MLL gene (1O1G1F).

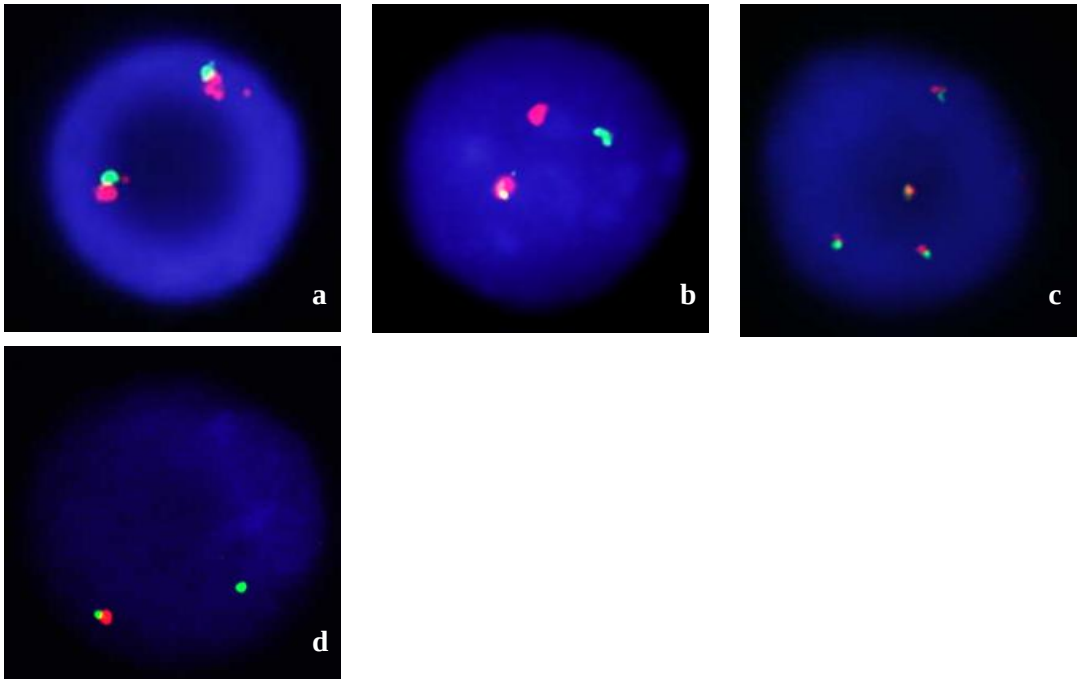

Figure 5.7 Split-signal FISH for detection of breaks in the IGH gene

a: An interphase cell with two fusion signals (IGH) are normal, no evidence of abnormality (2F).

b: An interphase cell with one orange signal, one green signal, and one fusion signal are split signal of IGH gene (1O1G1F).

c: An interphase cell with four fusion signals are four copies of IGH gene (4F).

d: An interphase cell with one green signal and one fusion signal are split signal of IGH gene with 3'IGH gene deletion (missing a orange signal from the cell) (1G1F).

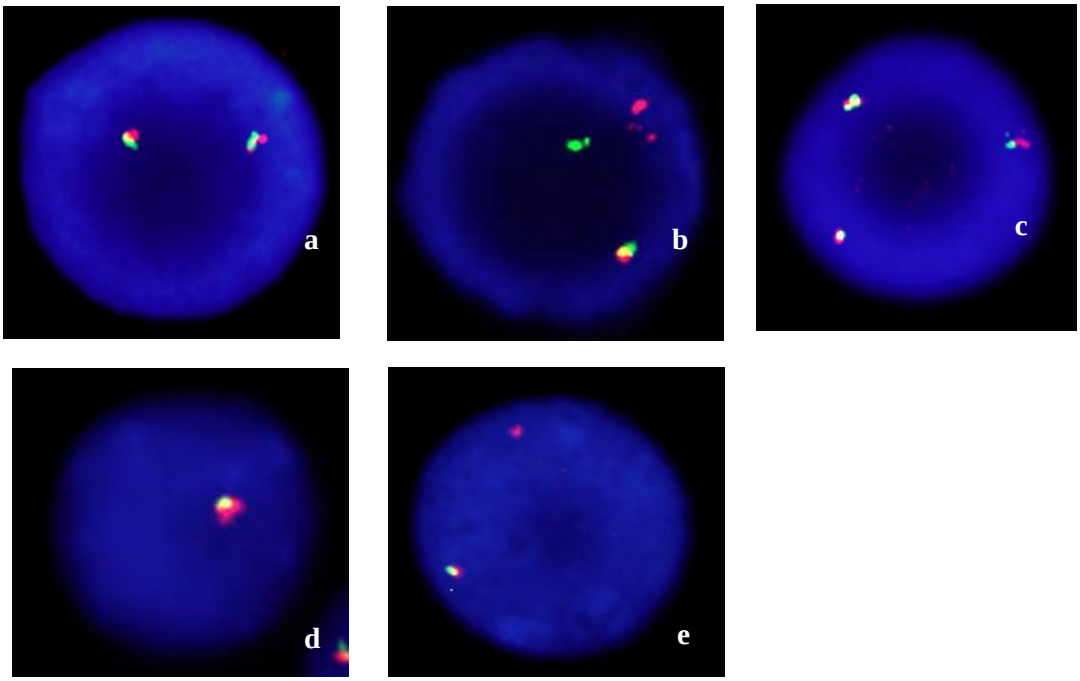

Figure 5.8 Split-signal FISH for detection of breaks in the PAX5 gene

a: An interphase cell with two fusion signals (PAX5) are normal (2F).

b: An interphase cell with one red signal, one green signal, and one fusion signal are split signal of PAX5 gene (1O1G1F).

c: An interphase cell with three fusion signals are three copies of PAX5 gene (3F).

d: An interphase cell with only one fusion signal indicating deletion of one PAX5 gene (missing a fusion signal from the cell) (1F).

e: An interphase cell with one green signal and one fusion signal are split signal of PAX5 gene with telomeric segment of PAX5 gene deletion (missing a green signal from the cell) (1O1F).

Table 5.3 Summary of Karyotype and I-FISH results in 32 ALL patients

Case no.	Karyotype	FISH							
		TEL/AML1(%)	ETV6(TEL)(%)	TCF3/PBX1(%)	TCF3(%)	BCR/ABL(%)	MLL(%)	IGH(%)	PAX5(%)
1	46, XY[1]	4O2G(98)						3F(75)	
2	56, XXX, +4, +6, +10, +14, +15, +17, +18, +21, +21, 1q?[6]	3O2G(15)						4F(99)	
3	46~47, XY, +1, +der(1)t(1;7)(p11;q11.2), der(1;14)(q10;q10), der(1;15)(q10;q10),der3t(1;3)(q11;p21), t(8;14)(q24.1;q32), der(11) dup(11)(q13q22 or q14q23) t(7;11)(q11.2;q25), der(11)dup(11)(q13q22 or q14q23), t(17;11)(q11.2;q25) dup(7)(q11.2;q21or q21q22), add(19)(p13.3)[ep39]/ 46XY[1] =97.5%/2.5%							1O1G1F(56)	
4	46, XX								
5	46, XX[11]	2O1G1F(16) 2O1F(60)	1O1G1F(9) 1O1G(56)						
6	45~46, XX, -2, der(6)t(6;22)(q25;q11.2), +8, -9, add(10)(q26), der(11)t(1;11)(q21;q21), der(11)t(8;11)(q?13; q?21), del(13)(q22,q32), -20, add(22)(q11.2), +add(22); (q11.2), +der(?) t(1;?; 13)(q21;?;q12)[ep21]			3O2G(91)		1O1G2F(53) 1O2G2F(15)		1G1F(72)	1F(93)
7	60, XX, -Y, -1, -3, -11, -12, -13, -15, -15, i(17)(q10), -19, -20, +21[1]/ 46, XY[13] = 7.1%/ 92.9%	4O3G(72)	3F(77)			2O3G(85)		3F(76)	
8	46, XY[19]/ 54, XXY, +der(1)t(1;12)(p32;p13), +4, +6, +8, +14, +21, +21[2]	4O1G(41)	1F	3O2G(80)				3F(17)	
9	46, XY[7]								1O1F(91)

Table 5.3 Summary of Karyotype and I-FISH results in 32 ALL patients (continued)

Case no.	Karyotype	FISH							
		TEL/AML1(%)	ETV6(TEL)(%)	TCF3/PBX1(%)	TCF3(%)	BCR/ABL(%)	MLL(%)	IGH(%)	PAX5(%)
10	NA	3O2G(81)							
11	52~55, XY,+X, +6, -8, der(9)del(9)(p?22) add(9)(q?34), -12, -14, +15, -15, +17, +18, -20, +21, -22, inc[cp6]/46, XY[2] = 75%/25%	4O2G(96)							1F(36)
12	46, XY[8]	2O1G1F(5) 2O1F(25)	1O1G1F(3) 1O1G(73)						
13	46,XY,t(9;22)(q34;q11.2)[29]/46,XY[1] = 96.7%/3.3%					1O1G2F(98)			
14	46, XY[7]								
15	46, XY[30]	2O1F(56)	1O1G(55)						
16	46, XY[12]								1F(42)
17	NA	4O2G(71)				3O2G(10)		3F(63)	3F(24)
18	NA			2O1G1F(86)	1O1F(87)				
19	Unanalyzable								
20	NA								1O1G1F(22)
21	46, XY, t(4;13)(p16; q12)[9]/ 46, XY[2] = 81.8%/ 18.2%	3O1F(80)	1O1G(90)					1G1F(62)	
22	46, XY[5]	2O1G1F(25) 3O1G1F(10)	1O1G1F(28)						

Table 5.3 Summary of Karyotype and I-FISH results in 32 ALL patients (continued)

Case no.	Karyotype	FISH							
		TEL/AML1(%)	ETV6(TEL)(%)	TCF3/PBX1(%)	TCF3(%)	BCR/ABL(%)	MLL(%)	IGH(%)	PAX5(%)
23	46, XX[19]	2O1G1F(57)	1O1G1F(54)						
24	46, XX[7].	2O1F(84)	1O1G(97)						
25	46, XX, t(7;15)(q22;q15)[4]								
26	46 XY[28]	U	U	U	U	U	U	U	U
27	46, XX[2]								
28	46, XX[20]	2O1G1F(85)	1O1G1F(80)						
29	46, XX, t(4;11)[33]/92, idem x 2[1]						1O1G1F(90)		
30	Unanalyzable	2O1G1F(78)	1O1G1F(82)						
31	NA	2O1G1F(65)	1O1G1F(56) 1O1G(15)						
32	53~56, XY, +?X, +3, +6, +8, +10, +11, +13, 14, +17, +18, +19, +21/hyperdiploidy/46, XY	4O2G(83)				3O2G(7)		3F(59)	3F(12)

FISH data are genetic changes detected in the 8 probe showed percentage occurrence in parentheses.
NA, not applicable; O indicates orange or red signal; G, green signal; F, fusion signal; U, un identified

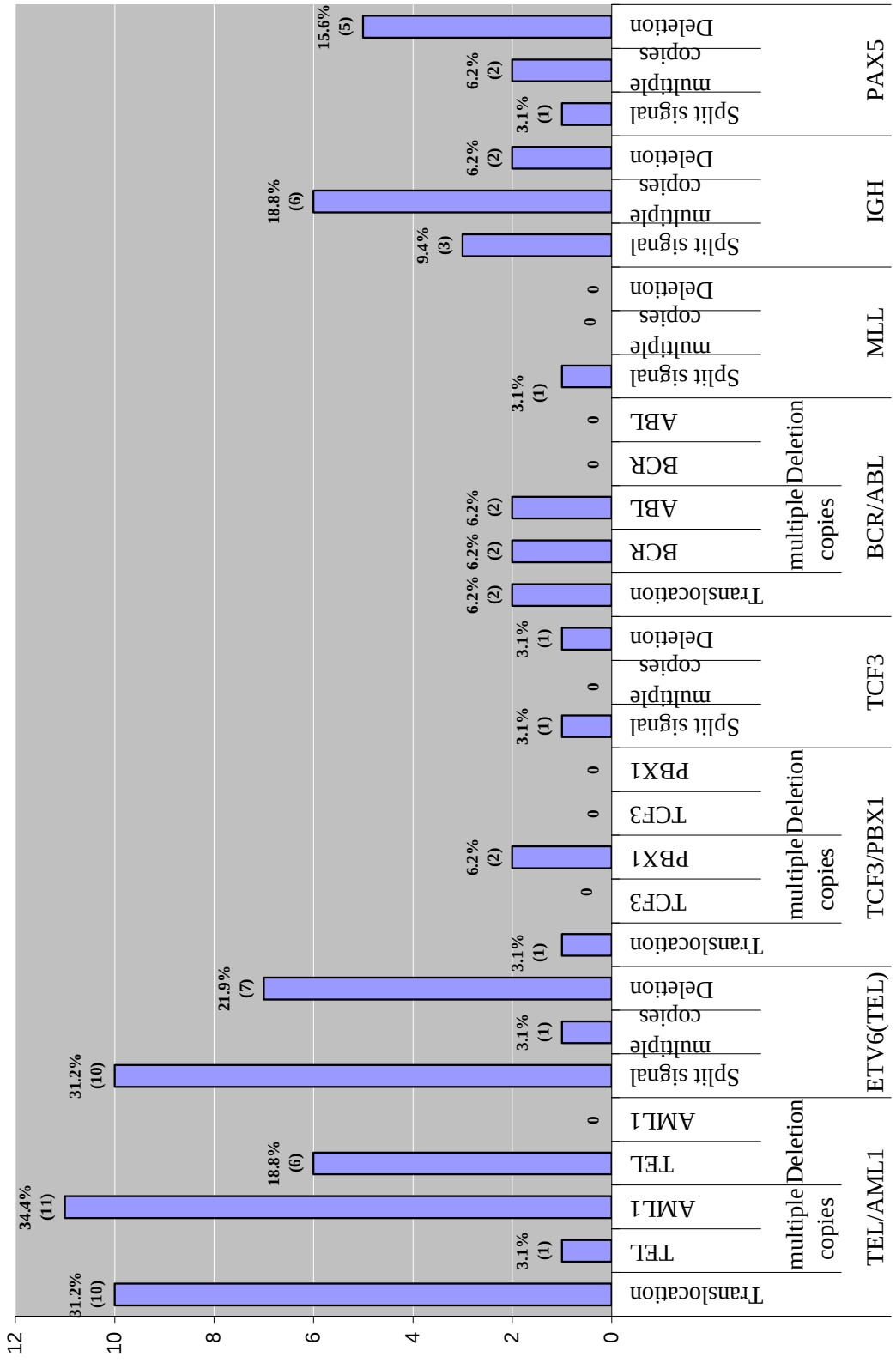

Figure 5.9 Frequencies and types of abnormalities identified by FISH analysis (data not include case no.26)

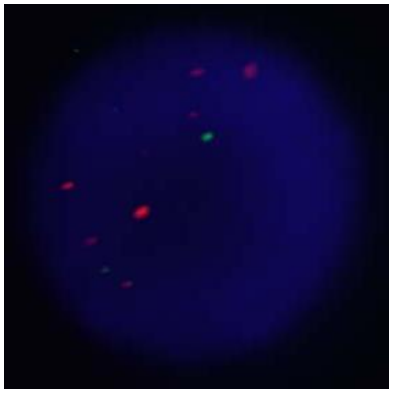

Figure 5.10 Amplification of AML1 gene. An interphase cell showed more than five orange signals (AML1 gene).

Table 5.4 Summary of the result with karyotype and FISH in 32 ALL patients

Karyotype (n=27)	FISH (n=32)	n
Normal	Normal	3
Abnormal	Abnormal	11
Normal	Abnormal	10
Abnormal	Normal	1
NA	Abnormal	5
Unanalyzable	Abnormal	1
	Normal	1

NA = not applicable

CHAPTER V
DISCUSSION

Current therapy for acute leukemia requires the assessment of both clinical and laboratory features to accurate assign patients to specific therapeutic subgroups. Non random chromosomal changes were found in more than 80% of patients with ALL and used as independent prognostic indicators (177, 178). Identification of genetic abnormalities are critical for diagnosis, risk stratification, and assessment of response to therapy in neoplastic disorders. Conventional cytogenetic remains the "gold standard" assay for detecting acquired karyotypic aberrations in oncology. However, this approach is limited by the availability of fresh tumor material, the growth characteristics of the tumor *in vitro*, and the mitotic index. Fluorescence *in situ* hybridization (FISH) can address these limitations by hybridizing appropriate DNA probes to complementary DNA sequences to detect specific cytogenetic aberrations in interphase nuclei (8, 9, 55, 179, 180). In our study, 32 childhood Thai ALL patients were also used I-FISH technique to detect gene rearrangements of B-lineage childhood ALL (BCP-ALL) by using TEL/AML1, ETV6(TEL), TCF3/PBX1, TCF3, BCR/ABL, MLL, IGH, and PAX5 probes TEL/AML1 fusion positive (31.4%) was the highest incidence of childhood Thai ALL patients which was similar to ZHANG Lijun, *et al.*'s study about 31.%. The TEL/AML1 fusion were capable to detect gene rearrangements by I-FISH technique whereas cytogenetic analysis showed normal karyotype or unanalyzed from metaphase cell (182). In case no.31, there was no result of karyotype, however, ETV6 split signal probe (TEL deletion) could detect unkown multiple partner genes that translocated with ETV6(TEL) gene which was uncorrelated with TEL/AML1 translocation probe. So, the usefulness for split signal probe were observed (183) which should be influence on prognosis. By previous study, patients with non-translocated ETV6 (TEL deletion) were classified into the high-risk group, whereas other patients with TEL/AML1 fusion alone were classified into low- or intermediate-risk group (179). In case no.26, the patterns of all available probes were

observed which related to bad prognosis in ALL patients (179). In case no.13, G-banding showed 46, XY, t(9;22)(q34;q11.2)[29]/46,XY[1] = 96.7%/3.3% and had correlated of the BCR/ABL translocation in FISH analysis. Our patient with 46, XX, t(4;11)[33]/92, idem x 2[1] in G-banding analysis had also translocated of the MLL gene in FISH (patients no.29). For PAX5 gene, majorities of PAX5 rearrangement were not found in G-banding because the breakpoints of these are too variable (185),however, 21.9% of PAX5 positive cases was found by FISH analysis, the previous reported revealed the split signal of PAX5 gene about 2.6% in BCP-ALL (184).Our study also had one case (3.1%) of split signal of PAX5 gene.

Summary of gene rearrangements in this study, TEL/AML1 gene with gain or loss of TEL or AML1 genes, the gains of the other genes without translocation, and the split signal of PAX5 gene alone were classified into low- or intermediate risk group whereas the others were classified into a high risk groups (186) in order to draw a conclusion concerning the correlation between clinical and laboratory findings, all patients in our study are closely followed up.

In overall study, gene rearrangements were identified in 87.5% of the patients using the combination of G-banding and FISH, while the chromosomal abnormalities were identified in 40.7% using G-banding alone. Gene rearrangements were disclosed by FISH in 27 (84.4%) of 32 patients with normal karyotype or no mitotic cell in G-banding. Especially, FISH is useful to identify the cryptic gene rearrangements in cases with normal banded karyotype or no mitotic cell in G-banding. The conventional G-banding analysis is able to identify the structural abnormalities in few patients with positive FISH results. In five cases that no evidence of abnormality in FISH result, only one case in these showed positive with karyotype : 46, XX, t(7;15)(q22;q15)[4] (case no.25), these might be due to the limitation of current commercial probes. Three cases had normal or unanalyzed with karyotype, this showed the gab of these two techniques. Thus, combination of testing methods that are both accurate and cost effective for each clinical situation has become important strategy.

Therefore, our data suggest that FISH testing using DNA probe specific for TEL/AML1, ETV6(TEL), TCF3/PBX1, TCF3, BCR/ABL, MLL, IGH, and PAX5 gene is a powerful tool for leukemia and it should be a routine procedure for all patients with newly diagnosed ALL. There will detect the majority of coexistent

chromosomal anomalies that may have a value in prediction treatment outcome, and prevention highly curable patients from experiencing unnecessary side effects of chemo/ radiation therapies.

CHAPTER VII
CONCLUSION

The World Health Organization (WHO) recent classification tumors of hematopoietic and lymphoid tissues emphasizes the importance of chromosome abnormalities for accurate diagnosis, appropriate treatment, and monitoring response to therapy. This study performed the FISH technique to screen 32 Thai childhood ALL patients by using translocation and breakapart probes to detect gene rearrangements such as TEL/AML1, TCF3/PBX1, BCR/ABl, ETV6 (TEL), TCF3, MLL, IGH, and PAX5 gene. FISH positivity was detected in 27 cases (84.4%) of all samples including 31.2% of TEL/AML1 translocation (case no.5, 12, 15, 21, 22, 24, 23, 28, 30 and 31), 3.1% of TCF3/PBX1 translocation (case no.18), 6.2% of BCR/ABL translocation (case no.6, and 13), 3.1% of MLL split signal (case no.29), 9.4% of IGH split signal (case no.3, 6, and 21), (3.1%) of PAX5 split signal (case no.20), and the gain or loss with or without translocated genes were also observed. This data demonstrated that FISH could investigate the higher percentage of genetic abnormalities and support the traditional diagnostic methodologies in childhood ALL. To summarize, FISH technique using DNA probes specific is a powerful tool to be used as a routine procedure for the detection of genetic abnormalities in the newly diagnosed of childhood ALL patients. The different in chromosomal aberrations could be used as a marker for the prognosis and treatment outcome.

REFERENCES

1. Miller R.W, Young J.L, Jr.&Novakovic B. Childhood cancer. Cancer. 1995;75:405-395.
2. Jaffe E.S, Harris N.L, Stein H, Vardiman J.W. Pathology and genetics of tumours of haematopoietic and lymphoid tissues, IARC Press, Lyon 2001.
3. Pui C. H. Childhood leukemias. N Engl J Med. 1995;332:1630-18.
4. Pui C-H, Crist W, Look A. Biology and clinical significance of cytogenetic abnormalities in childhood acute lymphoblastic leukemia. Blood. 1990;76:449-63.
5. M van der Burg, et al. Split-signal FISH for detection of chromosome aberrations in acute lymphoblastic leukemia. Leukemia. 2004;18:908-895.
6. Francesco Lo Cocort , et al. Southern Blot Analysis of ALL-1 Rearrangements at Chromosome 1lq23 in Acute Leukemia. CANCER RESEARCH. 1993;53:3803-0.
7. Pakasama S , et al. Simple multiplex RT-PCR for identifying common fusion transcripts in childhood acute leukemia. Int Jnl Lab Hem. 2008;30:291-86.
8. ZHANG Lijun, et al. Chromosomal changes detected by fluorescence in situ hybridization in patients with acute lymphoblastic leukemia
Chinese Medical Journal. 2003;116(9):1303-298.
9. Haas OA. Old and New Genetic Abnormalities in ALL and AML. StJude-Viva Forum. 2008:118-2.
10. Schultz KR, Pullen DJ, Sather HN, Shuster JJ, Devidas M, Borowitz MJ, et al. Risk- and response-based classification of childhood B-precursor acute lymphoblastic leukemia: a combined analysis of prognostic markers from the Pediatric Oncology Group (POG) and Children's Cancer Group (CCG). Blood. 2007;109: 935-26.
11. Greaves M.F, Maia A.T, Wiemels J.L, Ford A.M. Leukemia in twins: lessons in natural history. Blood. 2003;102:2333-21.

12. Maia A.T, Tussiwand R, Cazzaniga G, Rebulla P, Colman S, A B, et al. Identification of preleukemic precursors of hyperdiploid acute lymphoblastic leukemia in cord blood. Genes Chromosomes Cancer. 2004;40:43-38.
13. Mori H, Colman S.M, Xiao Z, Ford A.M, Healy L.E, Donaldson C, et al. Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc Natl Acad Sci USA. 2002;99:8247-2.
14. Stam R.W, den Boer M.L, Pieters R. Towards targeted therapy for infant acute lymphoblastic leukemia. Br J Haematol. 2006;132:551-39.
15. Greaves M. Speculations on the cause or childhood acute lymphoblastic leukemia. Leukemia. 1988;2:120-5.
16. Grignani F, Fagioli M, Alcalay M. Acute promyelocytic leukemia: From genetics to treatment. Blood. 1994(83):10-25.
17. Bennett JM, D. C, MT. D, et al. Proposals for the classification of the acute leukemias. French- American-British (FAB) co-operative group Br J Haematol. 1976;33(4):451-8.
18. Bennett JM, Catovsky D, Daniel MT, et al. The morphological classification of acute lymphoblastic leukemia: concordance among observers and clinical correlations. Br J Haematol 1981;47(4):553-61.
19. Kruse JR, Penchansky L, Contis L, Kaplan SS. Flow cytometry in the diagnosis of acute leukemia. Am J Clin Pathol 1988;89:341-6.
20. Preti A, Kantarjian H, Estey E. Characteristics and outcome of patients with acute lymphocytic leukemia and myeloperoxidase-positive blasts by electron microscopy. Hematol Pathol. 1994;8:155-67.
21. Ngan M, Chien K, Lee S. Sudan black B positivity in acute lymphoblastic leukemia. Mod Pathol. 1992;5:70-68.
22. Bowman W, Melvin S, Aur R, Mauer A. A clinical perspective on cell markers in acute lymphocytic leukemia. Cancer Res. 1981;41:4794-801.
23. Pilozzi E, Pulford K, Jones M, et al. Co-expression of CD79a (JCB117) and CD3 by lymphoblastic lymphoma. J Pathol. 1998;186(2):140-3.

24. Crist W, Grossi C, Pullen D, Cooper M. Immunologic markers in childhood acute lymphocytic leukemia. Semin Oncol. 1985;12:105-21.
25. Kurec A, Belair P, Stefanu C. Significance of aberrant immunophenotypes in childhood acute lymphoid leukemia Cancer 1991;67:3081-6.
26. Romana S, Mauchauffe M, Le Coniat M, Chumakov I, Le Paslier D, Berger R. The t(12;21) of acute lymphoblastic leukemia results in a TEL-AML1 gene fusion. Blood. 1995;85:3662-70.
27. Melsom H, Funderud S, Lie S, Godal T. The great majority of childhood lymphoblastic leukemias are identified by monoclonal antibodies and neoplasms of the B-cell progenitor compartment. Scand J Haematol. 1984;33:27-34.
28. Borowitz M, Shuster J, Civin C. Prognostic significance of CD34 expression in childhood B-precursor acute lymphocytic leukemia: A Pediatric Oncology Group study. J Clin Oncol ;:. 1990;8:1389-98.
29. Davis R, Longacre T, Cornbleet P. Hematogones in the bone marrow of adults. Immunophenotypic features, clinical settings and differential diagnosis. Am J Clin Pathol. 1994;102:202-11.
30. Koehler M, Behm F, Shuster J. Transitional pre-B-cell acute lymphoblastic leukemia of childhood is associated with favorable prognosis clinical features and an excellent outcome: A Pediatric Oncology Group study. Leukemia. 1993;7:2064-8.
31. Sulivan M, Pullen D, Crist W. Clinical and biologic heterogeneity of childhood B cell acute lymphocytic leukemia: Implications for clinical trials. Leukemia. 1990;4:6-11.
32. Preud'homme J, Brouet J, Danon F. Acute lymphoblastic leukemia with Berkitt's lymphoma cells: membrane markers and serum immunoglobulin. J Natl Cancer Inst. 1981;66:261-4.
33. Hammami A, Chan W, SD. M, Nassar V. Mature B-cell acute leukemia: A clinical, morphological, immunological and cytogenetic study of nine cases. Hematol Pathol. 1991;5:109-18.
34. Harden E, Haynes B. Phenotypic and functional characterization of human malignant T cell. Semin Hematol. 1985;22:13-26.

35. Borowitz M, Dowell B, Boyette J. Monoclonal antibody definition of T cell acute leukemia. Blood. 1985;65:785-8.

36. Kita K, Miwa H, Nakase K. Clinical importance of CD7 expression in acute myelocytic leukemia.The Japan Cooperative Group of Leukemia/Lymphoma. Blood. 1993;81:2399-405.

37. Lanier L, Chang C, Spits H, Phillips J. Expression of cytoplasmic CD3 proteins in activated human adult natural killer (NK) cells and CD3 complexes in fetal NK cells.Implications for the relationship of NK and T lymphocytes. Blood. 1992;81:603-12.

38. Kurtzberg J, Waldmann T, Davey M. CD7+, CD4-, CD8- acute leukemia: A syndrome of malignant pluripotent lympho-hematopoietic cells. Blood. 1989;73:381-90.

39. Crist W, Shuster J, Falletta J. Clinical features and outcome in childhood T-cell leukemia-lymphoma according to stage of thymocyte differentiation: A Pediatric Oncology Group study. Blood. 1988;72:1891-7.

40. Kalwinsky D, Raimondi S, Schell M. Prognostic importance of cytogenetic subgroups in de novo pediatric acute nonlymphocytic leukemia. J Clin Oncol 1990;8:75-83.

41. Walters R, Kantarjian H, Keating M. The importance of cytogenetic studies in adult acute lymphocytic leukemia. Am J Med. 1990;89:579-87.

42. Ma S.K, Wan T.S, Chan L.C. Cytogenetics and molecular genetics of childhood leukemia. Hematol Oncol. 1999;17:105-91.

43. Heim S, Mitelman F. Cancer Cytogenetics. 2nd ed. Wiley-Liss, New York; 1995.

44. Mitelman F. ed. International System for Human Cytogenetic Nomenclature(1995) ISCN (1995). Basel Switzerland Karger; 1995.

45. Nordgren A. Hidden aberrations diagnosed by interphase fluorescence in situ hybridisation and spectral karyotyping in childhood acute lymphoblastic leukemia. Leuk Lymphoma. 2003;44: 2039-53.

46. Scurto P, Hsu R, Kane J, Williams W, Haney D, Conn W, et al. A multiplex RT-PCR assay for the detection of chaimeric transcripts encoded by the risk-stratifying translocations of pediatric acute lymphoblastic leukemia. Leukemia. 1998;12:2005-1994.

47. Rubnitz J, Shuster J, Land V, Link M, Pullen D, Camitta B, et al. Case-control study suggests a favorable impact of TEL rearrangement in patients with B-lineage acute lymphoblastic leukemia treated with antimetabolite-based therapy: a Pediatric Oncology Group study. Blood. 1997;89:1143-6.

48. Barnard DR, Kalousek DK, Wiersma SR, Lange BJ, Benjamin DR, Arthur DC, et al. Morphologic, immunologic, and cytogenetic classification of acute myeloid leukemia and myelodysplastic syndrome in childhood: a report from the Childrens Cancer Group. Leukemia. 1995;10:12-5.

49. Pui-CH, Campana D, Crist WM. Towards a clinically useful classification of acute leukemias. Leukemia 1995;9:2157-4.

50. Dewald G. Modern methods of chromosome analysis and their application in clinical practice. Clin Lab Ann. 1983;2:29-1.

51. Eckert C, Landt O, Taube T, Seeger K, Beyermann B, Proba J, et al. Potential of LightCycler technology for quantification of minimal residual disease in childhood acute lymphoblastic leukemia. Leukemia. 2000;14:316-23.

52. Drunat S, Olivi M, Brunie G, Grandchamp B, Vilmer E, Bièche I, et al. Quantification of TEL-AML1 transcript for minimal residual disease assessment in childhood acute lymphoblastic leukemia. Br J Haematol. 2001;114:281-9.

53. Donovan J.W, Ladetto M, Zou G, Neuberg D, Poor C, Bowers D, et al. Immunoglobulin heavy-chain consensus probes for real-time PCR quantification of residual disease in acute lymphoblastic leukemia. Blood. 2000;95:2651-8.

54. Andreeff M, Pinkel D. Introduction to fluorescence in situ hybridization - principles and clinical applications.1st ed. Wiley-Liss, New York; 1999.

55. Kearney L. The impact of the new fish technologies on the cytogenetics of haematological malignancies. Br J Haematol. 1999;104: 648-58.

56. Speicher M.R, Gwyn Ballard S, Ward D.C. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet. 1996;12:368-75.

57. Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith M.A, et al. Multicolor spectral karyotyping of human chromosomes Science. 1996;273:494-7.

58. Raap A.K, Mutat Res. Advances in fluorescence in situ hybridization. 400. 1998:287-98.

59. Patel A.S, Hawkins A.L, Griffin C.A. Cytogenetics and cancer. Curr Opin Oncol. 2000;12:62-7.

60. van der Burg M, Beverloo HB, Langerak AW, Wijsman J v, Drunen E, Slater R, et al. Rapid and sensitive detection of all types of MLL gene translocations with a single FISH probe set. Leukemia. 1999;13:2113-07.

61. van Dongen JJM, Langerak AW. Molecular detection of chromosome aberrations (Split-signal FISH probe tchnology). International Patent. 1998;PCT/NL98/00270.

62. Werner M, Wilkens L, Aubele M, Nolte M, Zitzelsberger H, Komminoth P. Interphase cytogenetics in pathology: principles, methods, and applications of fluorescence in situ hybridization (FISH). Histochem Cell Biol. 1997;108:381-90.

63. Wolfe K.Q, Herrington C.S. Interphase cytogenetics and pathology: a tool for diagnosis and research. J Pathol. 1997;181:359-61.

64. Kallioniemi A, Kallioniemi O.P, Sudar D, Rutovitz D, Gray J.W, Waldman F, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science. 1992;258:818-21.

65. Forozan F, Karhu R, Kononen J, Kallioniemi A, Kallioniemi O.P. Genome screening by comparative genomic hybridization. Trends Genet. 1997;13:405-9.

66. Tachdjian G, Aboura A, Lapierre J.M, Viguié F, Ann Genet. Cytogenetic analysis from DNA by comparative genomic hybridization. 2000;43:147-54.

67. Alizadeh A.A, Ross D.T, Perou C.M, van de Rijn M. Towards a novel classification of human malignancies based on gene expression patterns. J Pathol. 2001;195: 52-41.

68. Pennisi E. Human Genome Project. And the gene number is...? Science. 2000;288:7-1146.

69. Claverie J.M. Gene number. What if there are only 30,000 human genes? Science. 2001;291: 7-1255.

70. Schena M, Shalon D, Davis R.W, Brown P.O. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. 1995;270:70-467.

71. Zhao N, Hashida H, Takahashi N, Misumi Y, Sakaki Y. High-density cDNA filter analysis: a novel approach for large-scale, quantitative analysis of gene expression. Genes Chromosomes Cancer. 1995;156:13-207.

72. Schena M, Shalon D, Heller R, Chai A, Brown P.O, Davis R.W. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc Natl Acad Sci U S A. 1996;93:10614-9.

73. Pevsner J. MICROARRAYS: GENOMEWIDE MEASUREMENT OF GENE EXPRESSION. Bioinformatics and Functional Genomics New Jersey: John Willey; 2009.

74. Jones D, Stapleton F, Kalwinsky D. Renal dysfunction and hyperuricemia at presentation and relapse of acute lymphoblastic leukemia. Med Pediatr Oncol 1990;18:283-6.

75. Ribeiro R, Abromowitch M, Raimondi S, Murphy S, Behm F, Williums D. Clinical and biologic hallmarks of the Philadelphia chromosome in childhood acute lymphoblastic leukemia. Blood. 1987;70:948-53.

76. Raimondi S, Behm F, Robertson P, Williums D, Pui C-H, Crist W, et al. Cytogenetics of pre-B-cell acute lymphoblastic leukemia with emphasis on prognosis implication of the t(1;19). J Clin Oncol 1990;8:1380-8.

77. Crist W, Carrol A, Shuster J, Behm F, Whitehead M, Vieti T, et al. Poor prognosis of children with pre-B acute lymphoblastic leukemia is associated with the t(1;19)(q23;p13). A pediatric Oncology Group Study. Blood. 1990;76:117-22.

78. Raimondi S, Peiper S, GR. K, Behm F, Williums D, Hancock M, et al. Childhood acute lymphoblastic leukemia with chromosomal breakpoints at 11q23. Blood. 1989;73:1627-34.

79. Pui C-H, Frankel L, Carroll A, Raimondi S, Shuster J, Head D, et al. Clinical characteristics and treatment outcome of childhood acute lymphoblastic leukemia with the t(4;11)(q21;q23): a collaborative study of 40 cases. Blood. 1991;77:440-7.

80. McLean T, Ringold S, Neuberg D, Stegmaier K, Tantravahi R, Ritz J, et al. TEL/AML-1 dimerizesand is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood. 1996;88:4252-8.

81. Greaves M, Janossy G, Peto J, Kay H. Immunologically defined subclasses of acute lymphoblastic leukemia in children: Their relationship to presentation features and prognosis. Br J Haematol. 1981;48:179-97.

82. Harousseau J, Tobelem G, Schaison G. High risk acute lymphocytic leukemia: A study of 141 cases with initial white blood cell counts over 100,000/cu. mm. Cancer 1980;46:2003-1996.

83. Sather H, Miller D, Nesbit M. Differences in prognosis for boys and girls with acute lymphoblastic leukemia. Lancet. 1981;1:739-43.

84. Sather H. Age at diagnosis in childhood acute lymphoblastic leukemia. Med Pediatr Oncol 1986;14:166-72.

85. Miller D, Leikin S, Albo V. Prognostic factors and therapy in acute lymphoblastic leukemia of childhood. Cancer. 1983;51:1041-9.

86. Santana V, Dodge R, Crist W. Presenting features and treatment outcome of adolescents with acute lymphoblastic leukemia. Leukemia. 1989;4:87-90.

87. Omura G, Moffitt S, Vogler W, Salter M. Combination chemotherapy of adult acute lymphoblastic leukemia with randomized central nervous system prophylaxis. Blood. 1989;55:204-199.

88. Hussein K, Dahlberg S, Head D. Treatment of acute lymphoblastic leukemia in adults with intensive induction, consolidation, and maintence chemotherapy. Blood. 1989;73:63-57.

89. Sather H, Coccia P, Nesbit M. Disappearance of the predictive value of the prognostic variables in childhood acute lymphoblastic leukemia. Cancer. 1981;48:370-6.

90. Omura G, Raney M. Long-term survival in adult acute lymphoblastic leukemia. J Clin Oncol. 1985;3:1053-8.

91. Bearly R, Johnson S, Lister T. Acute lymphoblastic leukemia in adults: clinicopathological correlations with the French-American-British (FAB) co-operative group classification. Eur J Cancer. 1979;15:909-14.

92. Pui C-H. Childhood leukemias. N Eng J Med. 1995;332:1618-30.

93. Miller D, Krailo M, Bleyer W. Prognostic implications of blast cell morphology in childhood acute lymphoblastic leukemia: A report from the Children's Cancer Study Group. Cancer Treat Rep. 1985;69:1211-21.

94. Look A, Roberson P, Williams D. Prognosis importance of blast cell DNA content in childhood acute lymphoblastic leukemia. Blood. 1985;65:1079-86.

95. Zippin C, Cutler S, Reeves W, Lum D. Variation in survival among patients with acute lymphocytic leukemia. Blood 1971;37:72-59.

96. Eden O, Rankin A, Kay H. Isolated testicular relapse in disease in acute lymphoblastic leukemia of childhood. Arch Dis Child. 1983;58:321-28.

97. Lampert F. Acute lymphoblastic leukemia: Current status of therapy in children. Cancer Res. 1984;93:159.

98. Zusman J, Brown D, Nesbit M. Hyperphosphatemia, hyperphosphateuria and hypocalcemia in acute lymphoblastic leukemia. N Engl J Med. 1973;289:1335-40.

99. Foon K, Todd R. Immunologic classification of leukemia and lymphoma. Blood. 1986;68:1-31.

100. Pui C-H, Behm F, Crist W. Clinical and biologic relevance of immunologic marker studies in childhood acute lymphoblastic leukemia. Blood. 1993;82:343-62.

101. Ritz J, Nadler L, Khan A. Expression of common acute lymphoblastic leukemia antigen (CALLA) by lymphomas of B-cell and T-cell origen. Blood. 195881;58:648-52.

102. Moretta L, Mingari M, Moretta A, Fauci A. Human lymphocyte surfacemarkers. Semin Hematol. 1982;19:273-84.

103. Vogler L, Crist W, Bockman D. Pre-B-cell leukemia: A new phenotype of childhood lymphoblastic leukemia. N Engl J Med. 1978;298:872-8.

104. Hoelzer D, Thiel E, Loffler H. Intensified therapy in acute lymphoblastic and acute undifferentiated leukemia in adults. Blood. 1984;64:38-47.

105. Raimondi S. Current status of cytogenetic research in childhood acute lymphoblastic leukemia. Blood. 1993;81:2237-51.

106. Clarkson B, Ellis S, Bittle C. Acute lymphoblastic leukemia in adults. Semin Oncol 1985;12:160-79.

107. Borowitz M. Immunological markers in childhood acute lymphoblastic leukemia Hematol Oncol Clin North Am 1990;4:743-65.

108. Crist W, Boyett J, Roper M. Pre-B cell leukemia responds poorly to treatment: A Pediatric Oncology Group study. Blood. 1984;63:407-14.

109. Carroll A, Crist W, Parmley R, Roper M, Cooper M, Finley W. Pre-B cell leukemia associated with chromosome translocation 1;19. Blood. 1984;63:721-4.

110. Pui C-H, Williams D, Kalwinsky D. Cytogenetic features and serum lactic dehydrogenase level predict a poor treatment outcome for children with pre-B-cell leukemia. Blood. 1986;67:1688-92.

111. Gill P, Meyer P, Pavlova Z, Levince A. B cell acute lymphocytic leukemia in adults: Clinical, morphologic, and immunologic findings. J Clin Oncol 1986;4:737-43.

112. Reiter A, Schrappe M, Ludwig W. Favorable outcome of B-cell acute lymphoblastic leukemia in childhood: A report of three consecutive studies of the BFM group. Blood. 1992;80:2471.

113. Sobol R, Royton I, LeBien T. Adult acute lymphoblastic leukemia phenotypes defined by monoclonal antibodies. Blood. 1985;65:730-5.

114. Greaves M, Pegram S, Chan L, Leuk Res Collaborative group study of the epidemiology of acute lymphoblastic leukemia subtypes: Background and first report. 1985;9:715-33.

115. Pui C-H, Behm F, Singh B. Heterogeneity of presenting features and their relation to treatment outcome in 120 children with T-cell acute lymphoblastic leukemia. Blood. 1990;75:174-9.

116. Sallen S, Ritz J, Pesando J. Cell surface antigens: Prognostic implications in childhood acute lymphoblastic leukemia. Blood. 1980;55:395-402.

117. Katsuno M, Abe Y, Taguchi F. CD7+ stem cell leukemia/lymphoma: Features of a subgroup without circulating blast cells. Cancer. 1993;72:99-104.

118. Yumura-Yaki K, Hara J, Kurahashi H. Clinical significance of CD7-positive stem cell leukemia: A distinct subtype of mixed lineage leukemia. Cancer. 1991;68:2273-80.

119. Pui C-H, Raimondi S, Head D. Characterization of childhood acute leukemia with multiple myeloid and lymphoid markers at diagnosis and at relapse. Blood. 1991;78:1327-37.

120. Wiersma S, Ortega J, Sobel E, Weinberg K. Clinical importance of myeloid antigen expression in acute lymphoblastic leukemia of childhood. N Engl J Med. 1991;324:800-8.

121. Sobol R, Mick R, Royston I. Clinical importance of myeloid antigen expression in adult acute lymphoblastic leukemia. N Engl J Med. 1987;316:1111-7.

122. Pui C-H, Hancock M, Head D. Clinical significance of CD34 expression in childhood acute lymphoblastic leukemia. Blood. 1993;82:889-94.

123. Behm F, Raimondi S, Schell M. Lack of CD45 antigen on blast cells in childhood acute lymphoblastic leukemia is associated with chromosomal hyperdiploidy and other favorable prognostic features. Blood. 1992;79:1011-6.

124. Uckun F, Gajl-Peczalska K, Provisor A, Heerema N. Immunophenotype karyotype associations in human acute lymphoblastic leukemia. Blood. 1989;73:271-80.

125. Pui C-H, Williams W, Robertson P. Correlation of karyotypes and immunophenotype in childhood acute lymphoblastic leukemia. J Clin Oncol. 1988;6:56-61.

126. Trueworthy R, Shuster J, Look A. Ploidy of lymphoblasts is the strongest predictor of treatment outcome in B-progenitor cell acute lymphoblastic leukemia of childhood: A Pediatric Oncology Group studt. J Clin Oncol. 1992;10:606-13.

127. Copelan E, McGuire E. The biology and treatment of acute lymphoblastic leukemia in adults. Blood. 1995;85:1151-68.

128. Viehmann S, Borkhardt A, Lampert F, Harbott J. Multiplex PCR-a rapid screening method for detection of gene rearrangements in childhood acute lymphoblastic leukemia. Ann Hematol. 1999;78:157-62.

129. Raynaud S, Cave H, Baens M, Bastard C, Cacheux V, Grosgeorge J. The 12;21 translocation involving TEL and deletion of the other TEL allele: two

frequently associated alterations found in childhood acute lymphoblastic leukemia. Blood 1996;87:2891-9.

130. Raynaud S, Mauvieux L, Boucheix C, Charrin C, Fiere D, Gabert J. TEL/AML1 fusion gene is a rare event in adult acute lymphoblastic leukemia. Leukemia. 1996;10:1529-30.
131. Loh M, Silverman L, Young M, Neuberg D, Golub R, Sallan S, et al. Incidence of TEL/AML1 fusion in children with relapsed acute lymphoblastic leukemia. Blood. 1998;92:4792-7.
132. Seeger K, Adams H, Buchwald D, Beyermann B, Kremens B, Niemeyer C, et al. TEL/AML1 fusion transcript in relapsed childhood acute lymphoblastic leukemia. Blood. 1998;91:1716-22.
133. Baens M, Peeters P, Guo C, Aerssens J, Marynen P. Genomic organization of TEL: the human ETS-variant gene 6. Genome Res. 1996;6:404-13.
134. Michael P, Levin M, Garson O. Translocation 1;19 - a new cytogenetic abnormality in acute lymphocytic leukemia. Cancer Genet Cytogenet. 1984;12:333-41.
135. Neale G, Menarguez J, Kitchingman G. Detection of minimal residual disease in T-cell acute lymphoblastic leukemia using polymerase chain reaction predicts impending relapse. Blood. 1991;78:739-47.
136. Bloomfield C, Goldman A, Alimena G, Berger R, Borgstrom G, Brandt L. Chromosomal abnormalities identify high-risk and low-risk patients with acute lymphoblastic leukemia. Blood. 1986;67:415-20.
137. Maurer J, Jansen J, Thiel E, Van Denderen J, Ludwig W, Audemir U. Detection of chimeric BCR-ABL genes in acute lymphoblastic leukemia by the polymerase chain reaction. Lancet 1991;337:1055-8.
138. Hermans A, Heisterkamp N, Von L M, Van Baal S, Van der Plas D, Wiedemann L. Unique fusion of bcr and c-abl genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell. 1987;51:33- 40.
139. Kurzrock R, Gutterman J, Talpaz M. The molecular genetics of Philadelphia chromosome-positive leukemias. New Engl J Med. 1988;319:990-8.
140. Shtivelman E, Lifshitz B, Gale R, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukemia. Nature. 1985;315:550-4.

141. Clark S, McLaughlin J, Crist W, Champlin R, Witte O. Unique forms of the abl tyrosine kinase distinguish Ph1-positive CML from Ph1-positive ALL. Science. 1987;253:8-85.

142. Van Rhee F, Hochhaus A, Lin F, Melo V, Goldman J, Cross N. p190 BCR/ABL mRNA is expressed at low levels in p210-positive chronic myeloid and acute lymphoblastic leukemias. Blood. 1996;87:5213-7.

143. Westbrook C, Hooberman A, Spino C. Clinical significance of the BCR-ABL fusion gene in adult acute lymphoblastic leukemia: A Cancer and Leukemia Group B study (8762). Blood. 1992;80:2983-90.

144. Preudhomme C, Henic N, Cazin B, Lai J, Bertheas M, Vanrumbeke M. Good correlation between RT-PCR analysis and relapse in Philadelphia (Ph1)-positive acute lymphoblastic leukemia (ALL). Leukemia 1997;11:294-8.

145. Radich J, Gehly G, Lee A, Avery R, Bryant E, Edmands S, et al. Detection of bcr-abl transcripts in Philadelphia chromosome-positive acute lymphoblastic leukemia after marrow transplantation. Blood. 1997;89:2602-9.

146. Borowitz M, Hunger S, Carroll A, Shuster J, Pullen J, Steuber C. Predictability of the t(1;19)(q23;p13) from surface antigen phenotype: implication for screening cases of childhood acute lymphoblastic leukemia for molecular analysis: a Pediatric Oncology Group Study. . Blood. 1993;83:1086-91.

147. Kamps M, Murre C, Sun X, Baltimore D. A new homeobox gene contributes the binding domain of the t(1;19) translocation protein in pre-B ALL. Cell. 1990;60:547-55.

148. Izraeli S, Kovar H, Gadner H, Lion T. Unexpected heterogeneity in E2A/PBX1 fusion messenger RNA detected by the polymerase chain reaction in pediatric petients with acute lymphoblastic leukemia. Blood. 1992;80:1413-7.

149. Troussard X, Rimokh R, Valensi F, Leboeuf D, Fenneteau O, Guitard A, et al. Heterogeneity of t(1;19)(q23;p13) acute leukemias. Br J Haematol. 1995;89:516-26.

150. Domer P, Fakharzadeh S, Chen C, Jockel J, Johansen L, Silverman G. Acute mixed-lineage leukemia t(4;11)(q21;q23) generates an MLL-AF4 fusion product. Proc Natl Acad Sci USA 1993;90:7884-8.

151. Ludwig W, Rieder H, Bartram C, Heinze B, Schwartz S, Gassmann W, et al. Immunophenotypic and genotypic features, clinical characteristics and treatment outcome of adult pro-B acute lymphoblastic leukemia: results of the German multicenter trials GMALL 03/87 and 04/89. Blood. 1998;92:1898-909.

152. Rubnitz J, Behm F, Pui C, Evans W, Relling M, Raimondi S, et al. Genetic studies of childhood acute lymphoblastic leukemia with emphasis on p16, MLL, and ETV6 gene abnormalities: results of St Jude Total Therapy Study XII. Leukemia. 1997;11:1201-6.

153. Griesinger F, Elfers H, Ludwig W, Falk M, Rieder H, Harbott J, et al. Detection of HRX-FEL fusion transcripts in pre-B-ALL with an without cytogenetic demonstration of t(4;11). Leukemia. 1994:8.

154. Nilson I, Lochner K, Siegler G, Greil J, Beck J, Fey G, et al. Exon/intron structure of the human ALL-1 (MLL) gene involved in translocations to chromosomal region 11q23 and acute leukemias. Br J Haematol. 1996;93:966-72.

155. Harrison C, Cuneo A, Clark R, Johansson B, Lafage-Pochitaloff M, Mugneret F, et al. Ten novel 11q23 chromosomal partner sites. Leukemia. 1998;85:1151-68.

156. Blutters-Sawatzki R, Borkhardt A, Grathwohl J, Repp R, Rheinisch-Becker I, Bohle R, et al. Secondary acute myeloid leukemia with translocation (4;11) and MLL/AF4 rearrangement in a 15-year-old boy treated for common acute lymphoblastic leukemia 11 years earlier. Ann Hematol. 1995;70:31-5.

157. Johansson B, Moorman A, Hass O, Watmore A, Cheung K, Swanton S, et al. Hematologic malignancies with t(4;11)(q21;q23): a cytogenetic, morphologic, immunophenotypic and clinical study of 183 cases.European 11q23 Workshop. Leukemia. 1998;12:779-87.

158. Lochner K, Siegler G, Fuhrer M, Greil J, Beck J, Fey G, et al. A specific detection in the breakpoint cluster region of the ALL-1 gene is associated with acute lymphoblastic T-cell leukemias. Cancer Res. 1996;56:2171-7.

159. Schichman S, Caligiuri M, Gu Y, Strout M, Canaani E, Bloomfield C, et al. ALL-1 partial duplication in acute leukemia. Proc Natl Acad Sci USA. 1994;91:6236-9.

160. Schnittger S, Wormann B, Hiddemann W, Griesinger F. Partial tandem duplications of the MLL gene are detectable in peripheral blood and bone marrow of nearly all healthy donors. Blood. 1998;92:1728-34.

161. Nilson I, Reichel M, Ennas M, Greim R, Knorr C, Siegler G. Exon/ intron structure of the human AF-4 gene, a member of the AF-4/LAF-4/FMR-2 gene family coding for a nuclear protein with structural alterations in acute leukemia. Br J Haematol 1997;98:157-69.

162. Pui C-H, Kane J, Crist W. Biology and treatment of infant leukemias. Leukemia. 1995;9:762-9.

163. Rowley J. A novel consistent chromosome abnormality in chronic myelogenous leukemia detected by quinacrine fluorescence and Giemsa staining. Nature. 1973;243:290-3.

164. Pan J, Xue Y, Wu Y, Wang Y, Shen J. Dicentric (7;9)(p11;p11) is a rare but recurrent abnormality in acute lymphoblastic leukemia: a study of 7 cases. Cancer Genet Cytogenet. 2006;169:163-59.

165. Reid A.G, Seppa L, von der Weid N, Niggli F.K, Betts D.R. A t(12;17)(p13;q12) identifies a distinct TEL rearrangement-negative subtype of precursor-B acute lymphoblastic leukemia. Cancer Genet Cytogenet. 2006;165:64-9.

166. Strehl S, Konig M, Dworzak M.N, Kalwak K, Haas O.A. PAX5/ETV6 fusion defines cytogenetic entity dic(9;12)(p13;p13). Leukemia. 2003;17:1123-1.

167. Frohling S, Schlenk RF, Stolze I, et al. CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol. 2004;22:633-24.

168. Nutt S.L, Heavey B, Rolink A.G, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature. 1999;401:562-56.

169. Nutt S.L, Morrison A.M, Dorfler P, Rolink A, Busslinger M. Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. The EMBO Journal. 1998;17:2333-19.

170. Roberts W, Estrov Z, Ouspenskaia M. Measurement of residual leukemia during remission in childhood acute lymphoblastic leukemia. N Engl J Med. 1997;336:317-23.

171. Uckun F, Kersey J, Haake R. Pretransplantation burden of leukemic progenitor cells as a predictor of relapse after bone marrow transplantation for acute lymphoblastic leukemia. N Engl J Med. 1993;329:1296-301.

172. Negrin R, Blume K. The use of the polymerase chain reaction for the detection of minimal residual malignant disease. Blood. 1991;78:255-8.

173. Kalwinsky D, Roberson P, Dahl G. Clinical relevance of lymphoblast biologic features in children with acute lymphoblastic leukemia. J Clin Oncol. 1985;85:873-8.

174. Pui C-H, Behm F, Singh B. Myeloid-associated antigen expression lacks prognostic value in childhood acute lymphoblastic leukemia treated with intensive multiagent chemotherapy. Blood. 1990;75:202-198.

175. Fletcher J, Kimball VL, E. Prognostic implications of cytogenetic studies in an intensively treated group of children with acute lymphoblastic leukemia. Blood. 1989;74:2130-5.

176. Earle E, Choo K. Methods in Molecular Biology and In situ hybridization protocols. NJ. 1994;33:158-47.

177. Harrison CJ. The genetics of childhood acute lymphoblastic practleukemia. Best Pract Clin Haematol. 2000:427-39.

178. Srinivals T, Peter D, Aplan D, et al. Molecular biology of acute lymphocytic leukemia. Semin Oncol. 1997;24:45-6.

179. Hee Yeon Woo, Dae Won Kim, Hyosoon Park, Ki Woong Seong, Hong Hoe Koo, Sun Hee Kim. Molecular Cytogenetic Analysis of Gene

Rearrangements in Childhood Acute Lymphoblastic Leukemia. J Korean Med Sci. 2005;20:36-41.

180. Harrison CJ, Moorman AV, Barber KE, Broadfield ZJ, Cheung KL, Harris RL, et al. Interphase molecular cytogenetic screening for chromosomal abnormalities of prognostic significance in childhood acute lymphoblastic leukemia: a UK Cancer Cytogenetics Group Study. Br J Haematol. 2005 May;129(4):520-30.
181. McLean TW, Ringold S, Neuberg D, et al. TEL/ AML-1 dimerizes and associated with a favorable outcome in childhood acute lymphoblastic leukemia Blood. 1996;88:4252-8.
182. Romana S. P, Le Coniat M, Berger R. t(12;21): a new recurrent translocation in acute lymphoblastic leukemia. Genes Chromosomes and Cancer. 1994;9:186-91.
183. M van der Burg, TS Poulsen, SP Hunger, HB Beverloo, EME Smit, K Vang-Nielsen, et al. Split-signal FISH for detection of chromosome aberrations in acute lymphoblastic leukemia. Leukemia. 2004;18:895-908.
184. K Nebral, D Denk, A Attarbaschi, M Ko¨nig, G Mann, OA Haas, et al. Incidence and diversity of PAX5 fusion genes in childhood acute lymphoblastic leukemia. Leukemia. 2009;23:134–43.
185. Qian An, Sarah L. Wrighta, Zoe¨ J. Konn, Elizabeth Mathesond, Lynne Mintod, Anthony V. Moorman, et al. Variable breakpoints target PAX5 in patients with dicentric chromosomes: A model for the basis of unbalanced translocations in cancer. PNAS. 2008;105:17050–4.
186. Donadieu J, Auclerc MF, Baruchel A, et al. Critical studies of prognostic factors in childhood acute lymphoblastic leukemia: differrences in outcome are poorly explained by the most significant prognostic variables. Br J Haematol. 1998;102:729-39.

APPENDIX

1. Reagent for cell preparation

- **25XPBS**

Sodium chloride 100g, Potassium chloride 2.5g, diSodium hydrogen phosphate 12.8 g, Potassium di hydrogen phosphate 2.5 g, add steriled distilled water to bring final volume to 500 ml. Autoclave this solution. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated

- 1X PBS Solution

To prepare 1X PBS solution, follow the formula listed below;

NaCl	8.5	g
Na_2HPO_4	1.35	g
$NaH_2PO_4 \cdot H_2O$	3.45	g

All the listed chemicals are dissolved in 1,000 ml distilled water, and adjust the pH to 7.4 Dispense into aliquots and sterilized by steam autoclave.

- Hypotonic solution (0.56% KCl)

Mix thoroughly KCl 5.6 g in 1000 ml steriled distilled water. Filtrate and store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

- Fixative

To prepare solution, follow the formula listed below;

Absolute methanol (three part)	75	ml
Glacial acetic acid	25	ml

Prepare fresh before each use

2. Reagent for hybridization

- Alcohol series (ethanol solution)

From absolute ethanol solution, prepare 3 jars with a 70%, 85% and 100% ethanol, respectively. Store cover jars at room temperature or at 2-8^{o}C. Discard diluted buffer if cloudy in appearance.

- **20XSSC solution**

Mix thoroughly 175.3 g and Sodium acetate 88.2 g. Add steriled distilled water to bring final volume to 1 liter. Measure pH and adjust to pH 7.0 with HCl. Autoclave this solution. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

- 2XSSC solution

Mix thoroughly 100 mL 20XSSC (pH 7.0) with 800 ml steriled distilled water Measure pH and adjust to pH 7.0 $\pm$ 0.2 with NaOH. Add purified steriled distilled water to bring final volume to 1 liter. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

- Denaturation Solution (70% Formamide/ 2XSSC)

Mix thoroughly 70 mL ultrapure formamide, 10 mL 20XSSC (pH 7.0) and add steriled distilled water to bring final volume to 100 ml. Measure pH using pH indicator strips to verify pH is 7.0-8.0. Between uses, store at 2-8^{o}C. Discard after 7 days

- RNase A Stock and Working Solutions

Prepare a stock solution of 10 mg/ml RNase A (DNase free) in 2XSSC. If RNase is not labeled as DNase free, then place RNse solution in boiling water bath for 10 minutes to inactive DNase. Aliquot into 10 µl volumes and store at -20 ^{o}C in capped 1.0 ml tube. At time of use, prepare working RNase solution by thawing the stock RNase solution (10 mg/ ml) in the tube and directly adding 990 µl of 2XSSC pH 7.0-7.4 to the 10 µl contained within the tube. Vortex to mix. Do not freeze-thaw. Discard after use.

- Pepsin A Stock and Working Solutions

Prepare a 10% Pepsin stock solution by mixing 1 g pepsin in 10 ml water (prewormed to 37 ^{o}C). Aliquot into 35 µl volumes and store at -20 ^{o}C until ready to use. When ready to use prepare a 0.005% pepsin working solution by mixing 35 µl of the 10% pepsin stock into solution of 69.3 ml purified water and 0.7 ml 1.0 N HCl that has been heated to 37 ^{o}C in Coplin jar (using a 37 oC waterbath).

- Formaldehyde Fixation Solution

Mix together 47.5 ml of 1XPBS, 2.5 ml of 1M MgCl2 and 1.3 ml of 37% formaldehyde. Mix well. Place in a Coplin jar. Prepare fresh each day.

- 1N HCl

HCl 8.35 ml, add steriled distilled water to bring final volume to 100 ml. Store at dark room ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

- **1M MgCl2**

Mg Cl2 20.331 g , add purified H_2O to bring final volume to 100 ml. Autoclave this solution. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

2. Reagent for washing and detection

- **0.4XSSC/ 0.3%NP-40 Wash solution**

Mix thoroughly 100 mL 2XSSC (pH 5.3) add 1.5 ml of NP-40. Mix thoroughly until NP-40 is completely dissolved, add purified H_2O to bring final volume to 500 ml. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

- 2XSSC/ 0.1%NP-40 Wash solution

Mix thoroughly 50 ml 20XSSC (pH 7.0), 500 µl of NP-40 Measure. Add purified H_2O to bring final volume to 500 ml. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminate.

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