MOLECULAR ANALYSES OF HEMATOLOGICAL MALIGNANCIES

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF
BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

BY

Z. BUKET YILMAZ

AUGUST 1998

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ANNEME ve BABAMA

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science.

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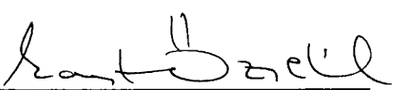

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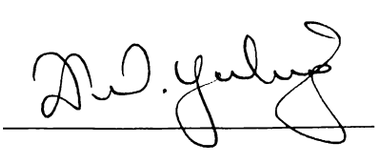

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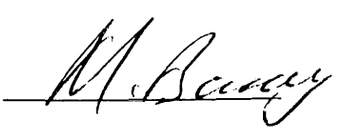

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ABSTRACT

Molecular Analyses of Hematological Malignancies

Z. Buket YILMAZ

M.Sc. in Molecular Biology and Genetics
Supervisor: Assoc. Prof. Dr. Tayfun Özçelik
August 1998, 140 pages

Normal hematopoiesis is known to be disrupted in hematological malignancies. As research advances, many new treatment techniques are introduced, among which allogeneic transplantation is the most effective. In patients who undergo such treatment, different hematopoietic chimeric states may result which can be detected by indirect analyses using DNA polymorphisms. MRD (minimal residual disease) describes leukemia cells present at a level below that is detectable by conventional means. The detection of MRD has gained special significance in transplantation patients. The detection of fusion transcripts that are generated by chromosomal rearrangements in various hematological malignancies is the direct and the most powerful approach for the detection of MRD. It not only has a prognostic significance but also has a diagnostic importance for patients who have not taken any treatment.

The aim of this thesis is the development of PCR-based tests for the diagnosis and monitoring of patients with different hematological malignancies. Therefore the chimerism status of 22 recipient-donor pairs have been evaluated by PCR amplification of STR and VNTR polymorphisms followed by polyacrylamide gel electrophoresis and silver staining. In addition, 44 patients with acute and chronic leukemias have been analyzed for the detection of fusion transcripts generated by t (9;22) (q34;q11), t (8;21) (q22;q22), t (15;17) (q22;q21), inv 16 (p13;q22), t (4;11) (q21;q23), and t (1;19) (q23;p23) chromosomal rearrangements with RT-PCR.

ÖZET

Hematolojik Kanserlerde Moleküler Analiz

Z. Buket YILMAZ

Yüksek Lisans Tezi, Moleküler Biyoloji ve Genetik Bölümü
Tez Danışmanı: Doç. Dr. Tayfun Özçelik
Ağustos 1998, 140 sayfa

Hematolojik kanserlerde normal hematopoezin zarara uğradığı bilinmektedir. Araştırmalardaki gelişmelere paralel olarak aralarında allojenik kemik iliği transplantasyonunun en etkili olduğu, birçok tedavi tekniği uygulamaya girmiştir. Bu tür tedavi alan hastalarda, DNA polimorfizmlerinin kullanıldığı dolaylı analiz yöntemleriyle tespit edilebilecek değişik kimerizm durumları görülmektedir. MRD (minimal residüel hastalık), standart analiz yöntemleriyle tespit edilebilecek seviyenin altındaki lösemi hücrelerinin varlığını ifade etmektedir. MRD analizi, transplantasyon hastalarında özel bir önem kazanmıştır. Değişik kan kanserlerinde kromozomlar arası parça değişimleriyle ortaya çıkan füzyon transkriptlerinin analizi, MRD tespitindeki doğrudan ve en etkili yöntemdir. Bu yöntem, prognostik özelliğinin yanı sıra, herhangi bir tedavi almamış hastalar için diagnostik açıdan da önem taşımaktadır.

Bu tezin amacı, hematolojik kanser hastalarında diagnostik ve prognostik amaçlı olarak PCR (polimeraz zincir reaksiyonu) yöntemine dayalı testlerin geliştirilmesidir. Bu amaca yönelik olarak, STR ve VNTR polimorfizmlerinin amplifikasyonu, bunu takiben poliakrilamid jel elektroforezi ve gümüş boyama yöntemleriyle 22 alıcı-verici çiftindeki kimerizm durumu değerlendirildi. Buna ek olarak, RT-PCR yöntemiyle t (9;22) (q34;q11), t (8;21) (q22;q22), t (15;17) (q22;q21), inv 16 (p13;q22), t (4;11) (q21;q23) ve t (1;19) (q23;p23) kromozomlar arası parça değişimleri sonucu ortaya çıkan füzyon transkriptlerinin analizi için, 44 akut ve kronik lösemi hastası incelendi.

ACKNOWLEDGEMENTS

First, and foremost I would like to express my gratitude to ***Assoc. Prof. Dr. Tayfun Özçelik*** for his support, friendly and kind-hearted policy, and for teaching that one is alone in life to overcome obstacles.

Special thanks go to ***Prof. Dr. Mehmet Öztürk*** for his invaluable intellectual contributions and advice whenever one is in need of. I also would like to thank ***Asst. Prof. Dr. Işık G. Yuluğ*** for her support, friendly approach and advice, and ***Prof. Dr. Ay Öğüş*** for her contributions.

I would like to thank ***Prof. Dr. Meral Beksaç*** and ***Dr. Celalettin Üstün*** for their contributions in the generation of this work.

Özlem Karaca is the one who deserves the most special thanks for just being herself and for being with me during the past thirteen years. I also thank my dear friend ***Dr. Tutku Soyer*** for enabling the unconditioned reliance and for showing that true friendship is forever. I thank ***Füsun Türesin***, ***Y. Senem Geylan***, and ***Seval Pehlivan*** for being real friends for the last six years and for making me feel very special.

I would like to thank ***Burçak Vural*** for her love, care and for being my elder sister; ***Alper Romano*** for all those scientific and other (!) discussions and deep understanding; ***Birsen Cevher*** for her valuable friendship; ***Hilal Özdağ*** and ***Lütfiye Mesci*** for sharing their experience; ***Cemaliye Akyerli*** and ***Arzu Atalay*** for making me feel in a family; ***Tuba Dinçer*** for making our house "*home*" full of joy and happiness and for sharing my problems patiently; ***Tolga Çağatay*** for his thoughtful, kind behaviours and support; ***Necati Fındıklı***, ***Çağla Eroğlu***, ***Korkut Vata***, and ***Emre Öktem*** for being my sweet friends; and ***Dr. Gökçe Törüner*** for his always available, valuable suggestions and comments.

Special thanks to ***Emre Sayan*** for making all impossible things possible for me, and to ***Berna Özçelik*** for her great support especially during the time of writing the thesis.

I would also like to thank ***Dr. Gürol Tunçman*** for all the joy we shared; and ***Füsun Elvan*** for providing my instant requests.

I would like to express my very special thanks to dear ***Reşat Ünal***, who made many things here worthwhile. Thanks for everything but particularly for being yourself, for never disappointing me and for your deep belief, love, care, and support I always felt.

I would also like to thank my other friends, and my family, whom I could not mention here one by one. I love you all.

Finally I would like to express my deepest gratitude to my precious mother ***Halime Çapkı***, and my dear father ***H. Orhan Yılmaz*** for their invaluable, unconditioned support, understanding, and endless love. You are the center of my life.

Thank you all from the heart for making everything worthwhile...

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ABBREVIATIONS

ALL	Acute lymphocytic leukemia
AML	Acute myelogenous leukemia
APL	Acute promyelocytic leukemia
APS	Ammonium persulphate
ATRA :	All-trans retinoic acid
BFU	Burst forming unit
BM	Bone marrow
BMT	Bone marrow tranplantation
bp	Base pair
β-TM :	Beta-thalassemia
CC	Complete chimeric
CFU	Colony forming unit
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CR	Complete remission
CSF	Cerebrospinal fluid
DEPC :	Diethylpyrocarbonate
DFS	Disease free survival
EDTA :	Ethylenediaminetetraacetate
FAB	French-American-British
FISH	Fluorescence *in situ* hybridization
GVHD:	Graft versus-host disease
GVL	Graft versus-leukemia
HLA	Human leukocyte antigen
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
kb	Kilobase
kD	Kilodalton
MC	Mixed chimeric
MDS	Myelodysplastic syndrome
MIC	Morphological immunological and cytogenetic
MOPS :	3-(N-morpholino) propanesulfonic acid
MRD	Minimal residual disease
NHL	Non-Hodgkin's lymphoma
p	Short arm of the chromosome
PAGE :	Polyacrylamide gel electrophoresis
PAH	Phenylalanine hydroxylase gene
PBSCT:	Peripheral blood stem cell transplantation
PCR	Polymerase chain reaction
Ph	Philadelphia
q	Long arm of the chromosome
R.E.A.L:	Revised European-American Classification of Lymphoid Neoplasms
RA	Retinoic acid
RAEB :	Refractory anemia with excess blasts
RAEB-t :	Refractory anemia with excess blasts in transformation
RAR	Retinoic acid receptor
RFLP :	Restriction length polymorphism
RT-PCR:	Reverse transcriptase - polymerase chain reaction
SDS	Sodium dodecyl sulphate
STR	Short tandem repeat
t	Translocation
TEMED:	N,N,N,N-tetramethyl-1,2 diaminoethane
VNTR :	Variable number of tandem repeats

I. INTRODUCTION

I. 1. HEMATOPOIETIC SYSTEM AND HEMATOLOGICAL MALIGNANCIES

1.1 Hematopoiesis : Genesis and Differentiation of Blood Cells

Hematopoiesis is the formation of blood cells (Fauci *et al.*, 1998). The bone marrow, lymph nodes, and spleen are all involved in hematopoiesis. These organs and tissues have traditionally been divided into *myeloid tissue* including the bone marrow and the cells derived from it- erythrocytes, platelets, granulocytes, and monocytes, and *lymphoid tissue* consisting of thymus, lymph nodes, and spleen (Cotran *et al.*, 1989).

Maximow, in 1924, postulated that blood cells were derived from a single class of progenitors. In 1938, Downey added the concept of hierarchies of pluripotent cells. The demonstration that single cells were capable of establishing nodules of hematopoietic growth in the spleen of irradiated mice and that such colonies displayed *multilineage* or *pluripotent* differentiation (erythroid, myeloid, megakaryocytic) came in 1961 by Till and McCulloch. These landmark experiments established that a *stem*

cell existed for hematopoiesis. A stem cell is a cell with the ability of self renewal and the production of progeny destined to differentiate (Lee *et al.*, 1993).

The common pluripotent hematopoietic stem cell gives rise to lymphoid stem cells and the trilineage myeloid stem cells respectively (Figure 1).

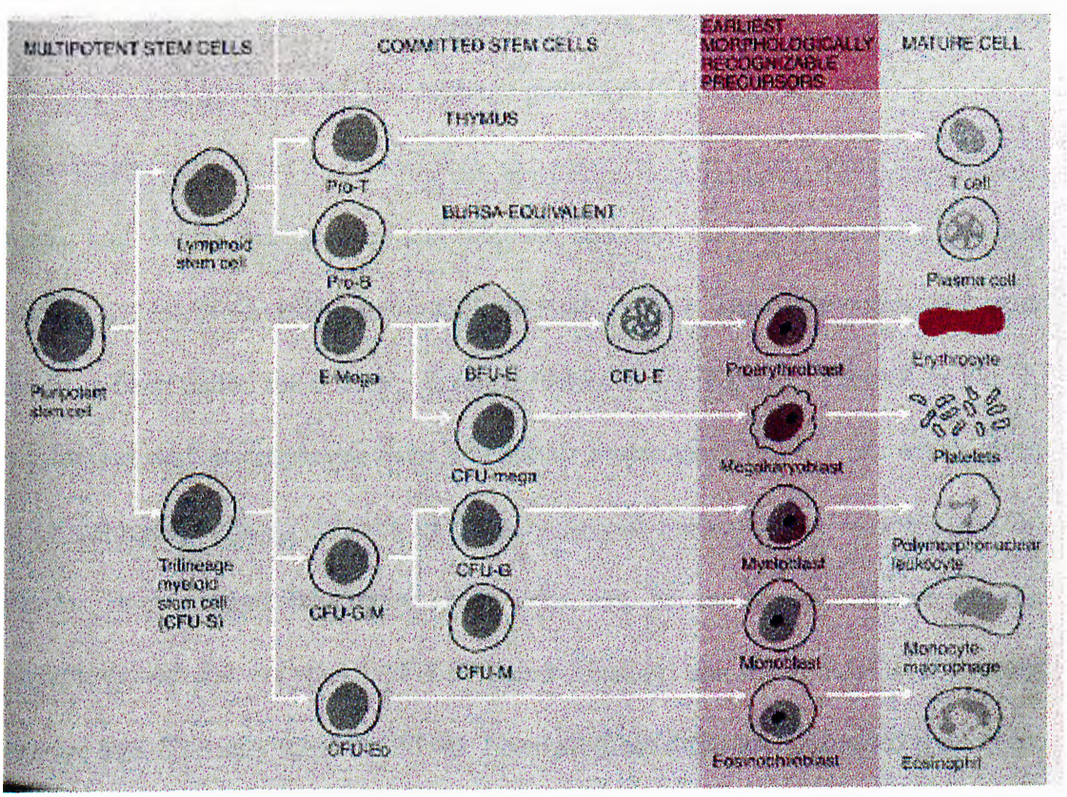

Figure 1 : **Differentiation of Hematopoietic Cells** (Cotran *et al.*, 1989)

The lymphoid stem cell is the origin of precursors of T cells (pro-T cells) and B cells (pro-B cells). The former differentiates into mature T cells under the inductive influence of the thymus and the latter to mature B cells under the influence of bursa-equivalent tissue. An important difference between lymphoid and myeloid differentiation is that, there are no distinctive, morphologically recognizable stages in lymphoid differentiation. From the multipotent myeloid stem cell, three types of *committed stem cells* arise which differentiate along the erythroid / megakaryocytic, eosinophilic, and granulocyte / macrophage pathways (Cotran *et al.*, 1989).

Pluznik and Sachs and Bradley and Metcalf reported that hematopoietic colonies could be grown in semisolid medium (Lee *et al.*, 1993). Thus, the committed stem cells have been called the colony - forming units (CFU). As it is indicated in Fig. 1, granulocytes and macrophages have a common precursor, CFU-G / M, which gives rise to colonies having a mixture of neutrophils and macrophages. In the erythroid pathway, two distinct committed stem cells can be recognized. Based on the morphology of the colonies, BFU-E (burst-forming unit-erythroid) is the more primitive one. The later stage is CFU-E. From all these different committed stem cells, intermediate stages are derived, and finally the morphologically recognizable precursors of the differentiated cell lines are formed. These are, proerythroblasts, myeloblasts, megakaryoblasts, monoblasts, and eosinophiloblasts which in turn give rise to mature progeny . The mature blood elements have a finite life span and their numbers must be constantly replenished. Thus, self renewal is an important property

of stem cells. The pluripotent stem cells have the greatest capacity of self renewal, but normally most of them are not in cycle. Self renewal ability declines as commitment proceeds, but a greater fraction of the stem cells are found in cycle (Cotran *et al.*, 1989).

During the development of systems for the growth of hematopoietic colonies in vitro, it was understood that, in the absence of either serum or conditioned medium, hematopoietic colonies did not grow. It was then demonstrated that glycoproteins termed *colony-stimulating factors* (CSF) were necessary for granulocyte-macrophage colony growth (Lee *et al.*, 1993). Some hematopoietic growth factors and their characteristics are summarized in Table 1.

1.2 Description and Function of Bone Marrow

The bone marrow is a reservoir of stem cells which also provides a unique microenvironment for the proliferation and the differentiation of precursor cells. In addition, it regulates the release of fully differentiated cells into the circulation. Both structural (stromal) and humoral components of the bone marrow are involved in support of hematopoiesis (Cotran *et al.*, 1989).

The marrow is a highly organized and complex organ. It is a gelatinous material interspersed within trabecular bone, which is supported by the cylindric medullary bone. A complex network of vessels organized into repeating units supplies the marrow cavity. Venous sinusoids, which are surrounded by hematopoietic tissue in which blood cell formation takes place, is formed by the combination of small vessels that reenter the marrow cavity. The production of the various marrow cells takes place by a 'colonial' proliferation of islands of erythroid or granulocytic cells, rather than by random distribution. Sinusoids are formed before myeloid hematopoiesis begins in the fetus. The venous sinusoids which eventually flow into a central vein, thus seem to be essential for hematopoiesis. The stromal elements of the marrow have been proposed as major factors in the regulation of hematopoiesis mainly through the mechanism of various cytokines that they export (Lee *et al.*, 1993).

Table 1 : Hematopoietic Growth Factors and Some of Their Characteristics

Factor*	Cells Stimulated	Production Sources
CSF-1 (M-CSF)	Monocytes	Endothelial cells, monocytes, fibroblasts
GM-CSF	All granulocytes, megakaryocytes, erythrocytes, stem cells, leukemic blasts	T cells, endothelial cells, fibroblasts
G-CSF	Granulocytes, macrophages, endothelial cells, fibroblasts, leukemic blasts	Endothelial cells, placenta, monocytes
IL-3	Granulocytes, erythroid cells, multipotential progenitors, leukemic blasts	T cells
IL-4	B, T cells	T cells
IL-5	B cells, CFU-Eo	T cells
IL-6	B, T cells, CFU-GEMM, CFU-GM, BFU-E, macrophages, neural cells, hepatocytes	Fibroblasts leukocytes, epithelial cells
IL-7	B cells	Leukocytes
IL-8	T cells, neutrophils	Leukocytes
IL-9	BFU-E, CFU-GEMM	Lymphocytes
IL-11	B, T cells, CFU-GEMM, macrophages	Macrophages
Erythropoietin	CFU-E, BFU-E	Kidney, liver

*GM, granulocyte-macrophage; IL, interleukin; GEMM, granulocytes, erythroid cells, macrophages, megakaryocytes (Lee *et al.*, 1993).

1.3 Hematological Malignancies

Hematological malignancies arise as a clonal proliferation of one of the hematopoietic progenitor cells. The clinical manifestations of a particular malignancy depend on the stage of differentiation and lineage of the affected cell. The specific nature of the initial mutation and of subsequent mutations that may take place during clonal evolution is also critical. The abnormal clone of cells must possess either a growth advantage or a block in apoptosis and / or differentiation over the normal cells. In lymphocyte proliferation, a causative mechanism has been shown to be a block in *fas*-mediated apoptosis. Clonal analysis of mature blood cells indicates that many myeloproliferative and / or myelodysplastic disorders generally arise in the pluripotent stem cell (Fauci *et al.*, 1998). The relationship between clonality and transformation into acute leukemia is summarized in Figure 2.

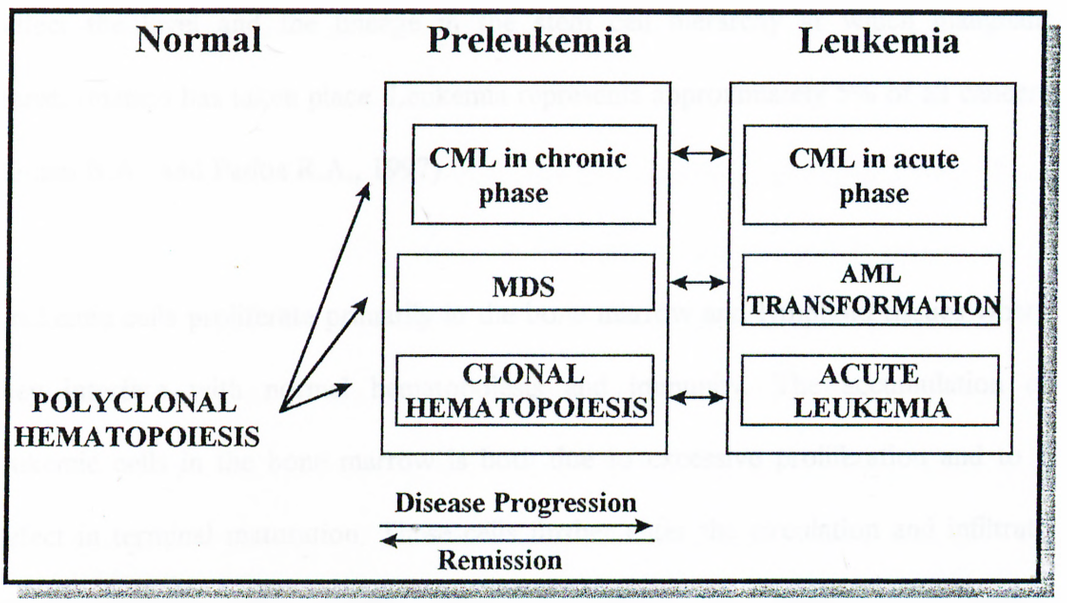

Figure 2 : Clonality and Transformation in Acute Leukemia

(Adapted from Lee *et al.*,1993)

1.3.1 Leukemias

The term ***leukemia*** was derived from the Greek word meaning 'white blood' and was first described by John Hughes Bennett and Rudolf Vichow in 1845 (Guinn B.A., and Padua R.A., 1997). The leukemias are a heterogeneous group of neoplasms that arise from the malignant transformation of hematopoietic cells (Wilson *et al.*, 1991). This group of cancers arise in immature hematopoietic cells and leukemias are constantly characterized by the disturbance of normal hematopoiesis and some degree of failure in production of normally functioning cells. The associated clinical features appear to

reflect the level and the lineage in the stem cell hierarchy at which malignant transformation has taken place. Leukemia represents approximately 5% of all cancers (Guinn B.A., and Padua R.A., 1997).

Leukemic cells proliferate primarily in the bone marrow and lymphoid tissues where they interfere with normal hematopoiesis and immunity. The accumulation of leukemic cells in the bone marrow is both due to excessive proliferation and to a defect in terminal maturation. These cells further enter the circulation and infiltrate into other tissues such as lymph nodes, liver, spleen, skin, viscera and the central nervous system (Wilson *et al.*, 1991).

Environmental toxins (e.g. benzene or other industrial chemicals), cancer chemotherapy, radiation, and viruses such as HTLV-I (human T-cell lymphotropic virus type-I in adult T-cell leukemia / lymphoma) are among the well-established leukemogenic factors. Several hereditary factors have also been implicated as significant risk factors in leukemia, especially in childhood. Most of them appear to be related to either some form of immune deficiency (agammaglobulinaemia, severe combined immunodeficiency, adenosinedeaminase deficiency), or a syndrome of chromosomal instability (Down syndrome) (Wilson *et al.*, 1991).

Disruption of Hematopoiesis in Leukemia

A limited number of hematopoietic stem cells sequentially enter into cell cycle. These then differentiate into multiple lineages in the peripheral blood and lymphoid organs. According to the traditional concepts of hematopoietic development, progenitor cells differentiate into a single phenotype without the ability to switch lineage. In general, leukemic transformation involves a particular lineage. However, some adult acute leukemias are "biphenotypic" in nature with the expression of both lymphoid and myeloid lineage cell surface antigens. In addition, in a subset of acute leukemias, lymphoid or myeloid lineage markers fail to be expressed and these are known as *acute undifferentiated leukemias*. Such leukemic cells may represent leukemic expansion of the stem cell itself with no lineage markers (Sawyers *et al.*, 1991).

Classification of Leukemias

The cell type involved and the state of maturity of the leukemic cells are the traditional bases on which leukemias are classified. Thus, *acute leukemias* are characterized by the presence of very immature cells, blasts, and by a rapid fatal course in untreated patients. On the other hand, *chronic leukemias* are at least initially associated with well-differentiated, mature, leukocytes and with a relatively slow course. There are two major variants of acute and chronic leukemias: *lymphocytic* and *myelocytic* (myelogenous). Therefore a simple classification yields four patterns of

leukemia: acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelocytic (myeloblastic) leukemia (AML), and chronic myelocytic leukemia (CML) (Cotran *et al.*, 1989).

FAB and MIC Classifications

In 1976, a uniform classification system for acute leukemias and myelodysplastic syndromes was developed by an international group of investigators. This system, known as the **FAB** (French-American-British) classification, is based on the morphologic appearance of bone marrow and blood leukemic blasts. This classification system has been adopted to allow comparison of treatment results and to take advantage of biologic differences between morphologic subtypes (Lee *et al.*, 1993).

A classification system based solely on morphological criteria proved to be inadequate. Therefore an additional system was introduced. Morphological, immunological and cytogenetic methods were integrated into this new classification by the **MIC** (Morphological, Immunological and Cytogenetic) Cooperative Study Group, but not all acute myeloid leukemias could be categorized by this classification as well (Segeren C. M., and van't Veer M. B., 1996).

Nevertheless, immunophenotyping can be helpful in confirmation of the diagnosis of AML and is necessary in differentiation of AML-Mo and AML-M7 from ALL (Table 2). However, it makes no contribution in distinguishing between AML and MDS. In this case, cytogenetic analysis will add important information as in MDS, numerical chromosomal abnormalities are more frequently observed while in *de novo* AML translocations are common. Immunophenotyping also is indispensable for the classification of acute undifferentiated leukemia which is not described in FAB classification. In the FAB classification, there is a strict discrimination between AML and ALL. However, less than 5% of leukemias are biphenotypic and in this case, this classification is lacking and immunophenotyping is essential for the recognition of these types of leukemia (Segeren C. M., and van't Veer M. B., 1996).

1.3.1.1 Acute Myeloblastic Leukemia (AML)

The term 'acute non-lymphocytic leukemia (ANLL) is also used for this group of leukemias. Acute myeloblastic leukemias primarily affect adults between ages of 15 and 39 years and constitute only 20% of childhood leukemias (Cotran *et al.*, 1989). The incidence of AML is approximately 2.3 per 100,000 people per year and it increases with age (Fauci *et al.*, 1998). The extraordinary heterogeneity of AML reflects the complexities of myeloid cell differentiation. This group of leukemias are of diverse origin. Some arise by transformation of multipotent (trilineage) myeloid stem

cells while in others the common granulocyte-monocyte precursor is involved (Fig 1).

In the widely used FAB classification, AML is divided into eight categories (see Table 2) taking into account both the degree of maturation (M1 to M3) and the predominant line of differentiation of the leukemic stem cells (M4 to M7) (Cotran *et al.*, 1989).

Table 2 : FAB Classification of Acute Myeloblastic (Myelocytic) Leukemias

Subtype		% of Cases *
Mo	Acute myeloblastic leukemia with no maturation	2-3 %
M1	Acute myeloblastic leukemia with minimal maturation	20 %
M2	Acute myeloblastic leukemia with maturation	25-30 %
M3	Acute promyelocytic leukemia	8-15 %
M4	Acute myelomonocytic leukemia	20-25 %
M5a	Acute monoblastic leukemia without differentiation	20-25 %
M5b	Acute monoblastic leukemia with differentiation	
M6	Acute erythroleukemia	5 %
M7	Acute megakaryocytic leukemia	1-2 %

* Percentages are approximations (Fauci *et al.*, 1998).

Chromosomal abnormalities have been observed in approximately 90 % of all AML cases. Many of the nonrandom chromosomal abnormalities have prognostic implications which do not depend on other clinical prognostic factors. These are summarized in Table 3.

Table 3 : Chromosomal Abnormalities in AML and Their Significance

Chromosome Abnormality	FAB Subgroup	Frequency	Comment
t (9;22) Ph chr.	M1	3 %	Poor to intermediate prognosis
t (8;21)	M2	20 %	More often in younger males, good prognosis
t (15;17)	M3	70-100 %	Unique to M3, intermediate prognosis
inv 16 or del 16q	M4	~ 25 %	Good prognosis
del 11q or t(11;V*)	M5, some M4	30 %	Intermediate prognosis
+8	M1, M2, M4, M5, M6	Variable	Common abnormality in hemotopoietic neoplasms, no prognostic association
-7 or del (7q) -5 or del (5q)	_	_	Most frequently seen in patients older than 60 years in secondary leukemias associated with exposure to environmental or occupational carcinogens

V* = Variable chromosomes (Cotran *et al.*, 1989)

1.3.1.2 Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukemia is primarily a disease of children and young adults. ALL can be subdivided by morphologic and immunologic criteria. Morphologic subtypes designated as L1, L2, and L3 have been defined in the FAB classification of acute leukemias. An alternative immunologic classification is also commonly used and is based on the origin of the leukemic lymphoblasts and their stage of differentiation. It is defined by cell surface markers and antigen receptor gene rearrangements (Cotran *et al.*, 1989).

Table 4 : Classification of Acute Lymphoblastic Leukemia (ALL)

Immunologic Subtype	% of Cases	FAB Subtype	Cytogenetic Abnormalities
Pre-B ALL	75	L1, L2	t (9;22), t (4;11), t (1;19)
T cell ALL	20	L1, L2	14q11 or 7q34
B cell ALL	5	L3	t (8;14), t (8;22), t (2;8)

(Adapted from Fauci *et al.*, 1998).

1.3.1.3 Chronic Myelogenous Leukemia (CML)

Chronic myelogenous leukemia (CML), is a clonal myeloproliferative disorder which results from the neoplastic transformation of the primitive hemopoietic stem cell. The disease is monoclonal in origin, affecting myeloid, monocytic, erythroid, megakaryocytic, B-cell, and sometimes T-cell lineages. CML is historically important in two aspects. First, it was the first disease in which a specific chromosomal abnormality t (9;22) (q34;q11) or *Philadelphia chromosome* was linked to the pathogenesis of the disease. Second is at the therapeutic level, CML is one of the first neoplastic diseases in which the use of a biologic agent, *interferon*, could suppress the neoplastic clone and prolong survival. Bone marrow transplantation (BMT) studies in CML have also given impressive results (Cortes *et al.*, 1996).

CML accounts for 7-15 % of all leukemias in adults with an incidence of 1-1.5 cases per 100,000 population. The median age at presentation is 53 years, but the disease can be seen in all age groups. The etiology of the disease is not clear. There is little evidence for genetic factors linked to CML. It has been suggested that there may be some correlation with HLA antigens CW3 and CW4. Therapeutic radiation has also been associated with increased risk of CML (Cortes *et al.*, 1996).

CML has a bi- or triphasic course. There is an initial chronic phase eventually leading to a blastic phase, which is sometimes preceded by an intermediate or accelerated

phase (Cortes *et al.*, 1996). Blast crisis is characterized by increased cellular proliferation, maturation arrest, and karyotypic clonal evolution (Wada *et al.*, 1994). A classification of CML and its variants is shown in Table 5.

Table 5 : A Classification of CML

Clinical Variants	Morphologic Variants
Typical CML (Ph chromosome present)	Chronic eosinophilic leukemia
Atypical CML (Ph chromosome absent)	Chronic basophilic leukemia
CML in infants	Chronic monocytic leukemia
	Chronic neutrophilic leukemia

(Lee *et al.*, 1993)

1.3.2 Lymphomas

The malignant lymphomas, in contrast to leukemias, are neoplastic transformations of cells that reside predominantly in lymphoid tissues. The two major variants of malignant lymphoma are *non-Hodgkin's lymphoma* and *Hodgkin's disease*. For the classification of the lymphoid malignancies, the Revised European-American Classification of Lymphoid Neoplasms (R.E.A.L.) has been created. (Fauci *et al.*, 1998).

Non-Hodgkin's lymphomas occur in the cases of drug-induced immunosuppression and acquired or congenital immunodeficiency. Such lymphomas develop in 30 % of HIV-1 infected individuals and 40-50 % are EBV (Epstein-Barr Virus) associated. At the molecular level, chromosomal translocations have been demonstrated to be involved in induction of non-Hodgkin's lymphoma. Genes that normally regulate heavy and light chain immunoglobulin synthesis are juxtaposed to genes that regulate normal cellular activation and proliferation. The postulation is that, these transforming oncogenes come under the control of those regulatory elements that normally control B cell proliferation and differentiation (Fauci *et al.*, 1998).

Hodgkin's disease usually presents as a localized disease with a subsequent spread to contiguous lymphoid structures and a final dissemination to non-lymphoid tissues. There is strong evidence for a role of EBV in the pathogenesis of the disease. In contrast to non-Hodgkin's lymphomas, where chromosomal deletions and translocations are common, cytogenetic studies have shown that such abnormalities are uncommon in Hodgkin's disease. Diagnosis depends on identification of large multinucleated reticulum cells (Reed-Sternberg cells) in lymph node tissue or other sites (Fauci *et al.*, 1998).

1.3.3 Myelodysplastic Syndromes (MDS)

Myelodysplastic syndromes, also referred to as preleukemic disorders, are a group of clonal acquired blood disorders affecting the hematopoietic stem cells and that often progress into acute leukemia. MDS results from neoplastic transformation of the pluripotent stem cell with involvement of myeloid, and less commonly, of lymphoid lineages. The myeloid cells in MDS have their ability to proliferate and differentiate, but they undergo an abortive maturation which results in an inadequate major blood cell production (Fauci *et al.*, 1998).

Among the common chromosomal abnormalities are, monosomy 7, 5q-, and trisomy 8 together with a variety of translocations. Molecular studies have demonstrated an increased incidence of mutations involving the *ras*, *cFms* [monocyte colony-stimulating factor (M-CSF) receptor] , *p53* and *RB* genes (Fauci *et al.*, 1998).

According to the FAB classification, there are five well-defined subtypes: *refractory anemia* (RA), *refractory anemia with ringed sideroblasts* (RARS), *refractory anemia with excess blasts* (RAEB), *refractory anemia with excess blasts in transformation* (RAEB-t), and *chronic myelomonocytic leukemia* (CMML) (Fauci *et al.*, 1998).
The information on the frequency and the leukemic transformation of these subtypes are summarized in Table 6. (See also Fig. 2).

Table 6 : FAB Classification and Leukemic Transformation in MDS

FAB Type	% of Cases	% Leukemic Transformation (Range)
RA	28	11 (0-20)
RARS	24	5 (0-15)
RAEB	23	20 (3-55)
CMML	16	23 (11-50)
RAEB-t	9	48 (11-75)

(Adapted from Fauci *et al.*, 1998)

1.4 Chromosomal Rearrangements in Hematological Malignancies

The first consistent chromosome aberration observed in human neoplasia was the Philadelphia chromosome in CML in 1960. The proof that this is a consequence of a translocation came in 1973 by the improved chromosome banding techniques. Translocations have one of two effects. They may lead to the *deregulation (overexpression) of oncogenes* by their juxtaposition to enhancer or promoter sequences that are active in the cell type from which the tumor arises, particularly the Ig and TCR enhancers. The alternative molecular consequence of translocations is

gene fusion, which results in a chimeric oncoprotein, the contribution to whose transforming ability is from both partners (Solomon *et al.*, 1991).

The frequently seen chromosomal rearrangements, which have also been studied in the scope of this thesis, involve five translocations and one inversion. Besides their frequent involvement in leukemias, the availability of samples for analysis was also taken into account. The rearrangements analyzed, their fusion gene products, disease association, affected age groups, and frequency in different leukemias is given in Table 7.

Table 7: Chromosomal Rearrangements Analyzed in the Scope of This Study

Rearrangement / Translocation	Fusion Gene	Disease	Age Group	Frequency
		CML; ALL;		90% (CML);5% (P),
t (9;22) (q34; q11)	*BCR / ABL*	AML (M1)	A / P	20% (A): 3% (M1)
t (8;21) (q22; q22)	*ETO / AML1*	AML (M2); APL	A / P	40% (P), 6% (A); 95%
t (15;17) (q22; q21)	*MYL / RAR-α*	AML (M3)	A / P	70-100%
t (4;11) (q21; q23)	*ALL-1 / AF-4*	ALL (lymhoblastic)	A / P	100%
t (1;19) (q23; p23)	*E2A / PBX-1*	ALL (pre-B cell)	P	20-25%
inv 16 (p13; q22)	*CBFB / MYH11*	AML (M4Eo)	A	~ 25%

A /P: Adult / Pediatric

1.4.1 t (9;22) (q34; q11)

The Ph chromosome is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22. In 1984, Groffen *et al.* located the breakpoints on chromosome 22 band q11 to a region of 5.8 kb, *the breakpoint cluster region* or bcr. Subsequent studies revealed that the bcr is part of a large gene called the *BCR* gene. The bcr contains four exons (b1-b4), and chromosomal breaks occur in two introns between exons b2, b3, and b4. Breakpoints on chromosome 9 are scattered over a distance of at least 100 kb, but are all located at the 5' end of the tyrosine kinase domain of the c-abl oncogene (Hermans *et al.* 1987).

The normal *BCR* gene occupies a region of about 135 kb on chromosome 22. It is expressed as mRNAs of 4.5 and 6.7 kb, which encodes for the same cytoplasmic 160-kD protein, and contains 23 exons. The Bcr protein contains a unique serine / threonine kinase activity and at least two SH2 binding sites encoded in its first exon and a C-terminal domain that functions as a GTPase activating protein (http://www.ncbi.nlm.gov/htbin-post/Omim/dispmim?151410). *BCR* first exon sequences specifically activate the tyrosine kinase and transforming potential of *BCR-ABL* through interaction of SH2 domain of ABL (Pendergast *et al.* 1991). The wild type Bcr has structural features that suggest a role in signal transduction. In addition to the novel serine / threonine kinase domain, it has an oligomerization domain, a Rho guanine-nucleotide exchange factor (Rho-GEF) homology domain, and a calcium-

dependent lipid binding site. *Bcr* knockout mice show defects in neutrophil superoxide bursts, suggesting a role for *Bcr* in the anti-microbial function of myeloid cells (Raitano *et al.*, 1997).

The *ABL* (Abelson strain of murine leukemia virus) gene is about 225 kb in size and is expressed as either a 6- or 7-kb mRNA transcript, with alternatively spliced first exons, exons 1a and 1b respectively. The 145 kD protein is classified as a nonreceptor tyrosine kinase (http://www.ncbi.nlm.gov/htbin-post/Omim/dispmim?189980). It contains src homology domains SH1, SH2, and SH3. Its carboxy-terminal has a number of important domains such as nuclear localization signals, a DNA binding domain, a p53 binding site, and an actin binding domain. Abl is expressed in all tissues and is localized in both nucleus and cytoplasm. Mice with targeted disruptions in the c-Abl gene have high neonatal mortality rates and increased susceptibility to infections suggesting a role in B-cell development, but its precise role is unclear (Raitano *et al.*, 1997).

As a result of the translocation, the *ABL* gene is transferred from its normal position on chromosome 9 band q34 to the Ph chromosome. This creates a head-to-tail *BCR-ABL* juxtaposition on the Ph chromosome. The fusion gene is transcribed into an 8.5 kb chimeric mRNA, lacking the first exon of *ABL*. The hybrid RNA is translated into a hybrid protein product of 210 kD ($p210^{BCR\text{-}ABL}$) and exhibits an in vitro tyrosine kinase activity . The Ph chromosome is also found in ALL (Ph+ ALL). Although *ABL*

is translocated and a new fusion protein $p190^{BCR-ABL}$ is formed, no breakpoints are observed in the bcr (Ph+ bcr- ALL). Breakpoints in chromosome 22 occur within the same gene but more 5' of the bcr. This fusion gene is transcribed into a 7 kb mRNA, encoding a novel fusion protein (Figure 3) (Hermans *et al.* 1987).

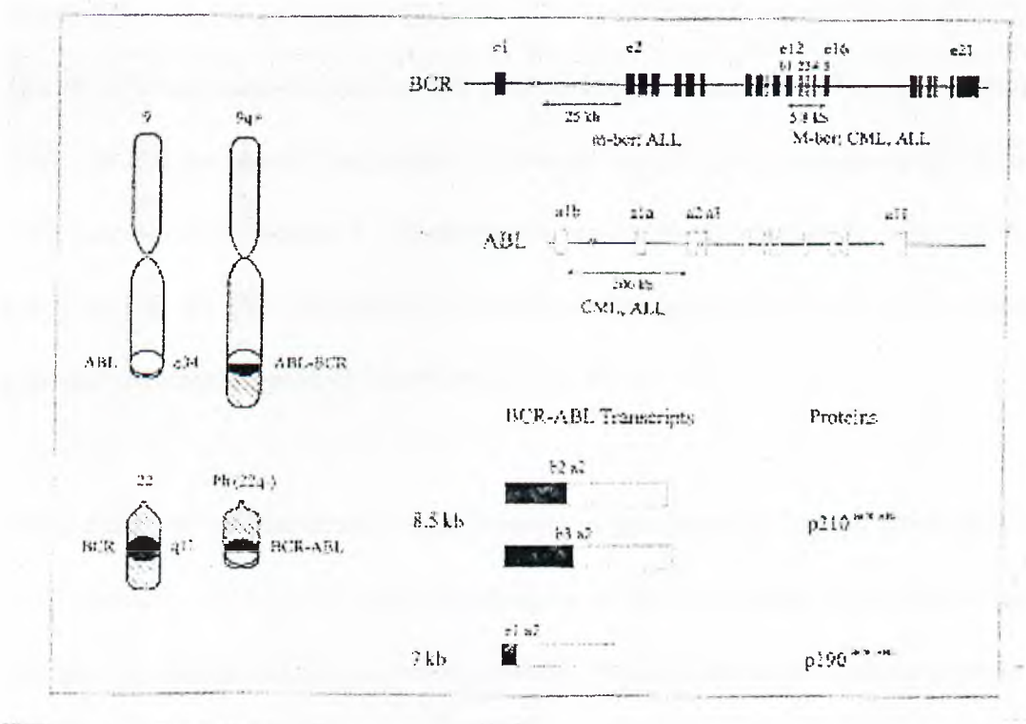

Figure 3: *BCR-ABL* Fusion in t (9;22) (q34; q11)

(Kurzrock R., and Talpaz M., 1995)

BCR-ABL transforms hematopoietic cells in vitro and its expression in growth-factor dependent cell lines allows cytokine independent growth. Lethally irradiated mice reconstituted with BCR-ABL expressing BM cells develop different hematological malignancies including a CML-like syndrome (Raitano *et al.*, 1997).

1.4.2 t (8;21) (q22;q22)

The 8;21 translocation is one of the most common chromosomal translocations in AML. The chromosomal breakpoints involve the *AML1* gene on chromosome 21 and *ETO* gene on chromosome 8. Translocation results in the consistent fusion of these genes on the der (8) chromosome, resulting in the production of a novel chimeric gene and message (Figure 4) (Downing *et al.*, 1993).

AML1 gene , on chromosome 21 q22, consists of nine exons and spans more than 150 kb of genomic DNA. It is highly homologous to the Drosophila segmentation gene *runt* and the mouse transcription factor pebp2 (polyoma enhancer binding protein) α subunit gene. The region of homology, called the Runt domain, is responsible for DNA binding and protein-protein interaction (Miyoshi *et al.*, 1995). To investigate the normal biologic function of *AML1 in vivo,* Okuda *et al.* generated mice carrying a disrupted *AML1* allele using gene targeting in embryonic stem (ES) cells. Mice lacking *aml1* died during midembryonic development, secondary to the complete absence of fetal-liver derived hematopoiesis. Besides, homozygous *aml1*-deficient

cells failed to contribute to hematopoiesis in chimeric animals indicating that, *aml1* regulated target genes are essential for definitive hematopoiesis of all lineages (Okuda *et al.*, 1996).

ETO ,also called *MTG8* (myeloid translocation gene on 8), contains two DNA-binding zinc finger motifs and several regions that are proline- and serine-rich. It is thought to be a transcription factor due to the presence of such motifs in other transcription factors (Nucifora G., and Rowley J. D., 1994). The relatively high levels of *ETO* in developing brain suggests that it could be involved in the regulation of some aspect of neuronal proliferation and differentiation (Erickson *et al.*, 1994). In a recent study by Wang *et al.*, *ETO* has been shown to have transforming properties. Ectopic *ETO* expression in NIH / 3T3 cells led to foci of transformation and colony growth in soft agar. *ETO*-expressing cells induced tumors following injection into irradiated nude mice suggesting an important role in the leukemogenic transforming potential of the *AML1-ETO* fusion protein (Wang *et al.*, 1997).

The fusion gene that results from the t (8;21) translocation contains the 5' region of *AML1* including the Runt domain fused to almost all of *ETO* (Nucifora G., and Rowley J. D., 1994). Both Aml1-Eto and the Aml1 proteins recognize the same consensus DNA-binding motif, which is found in promoters of several genes involved in hematopoiesis. *BCL-2* is another gene in which this sequence specific binding of

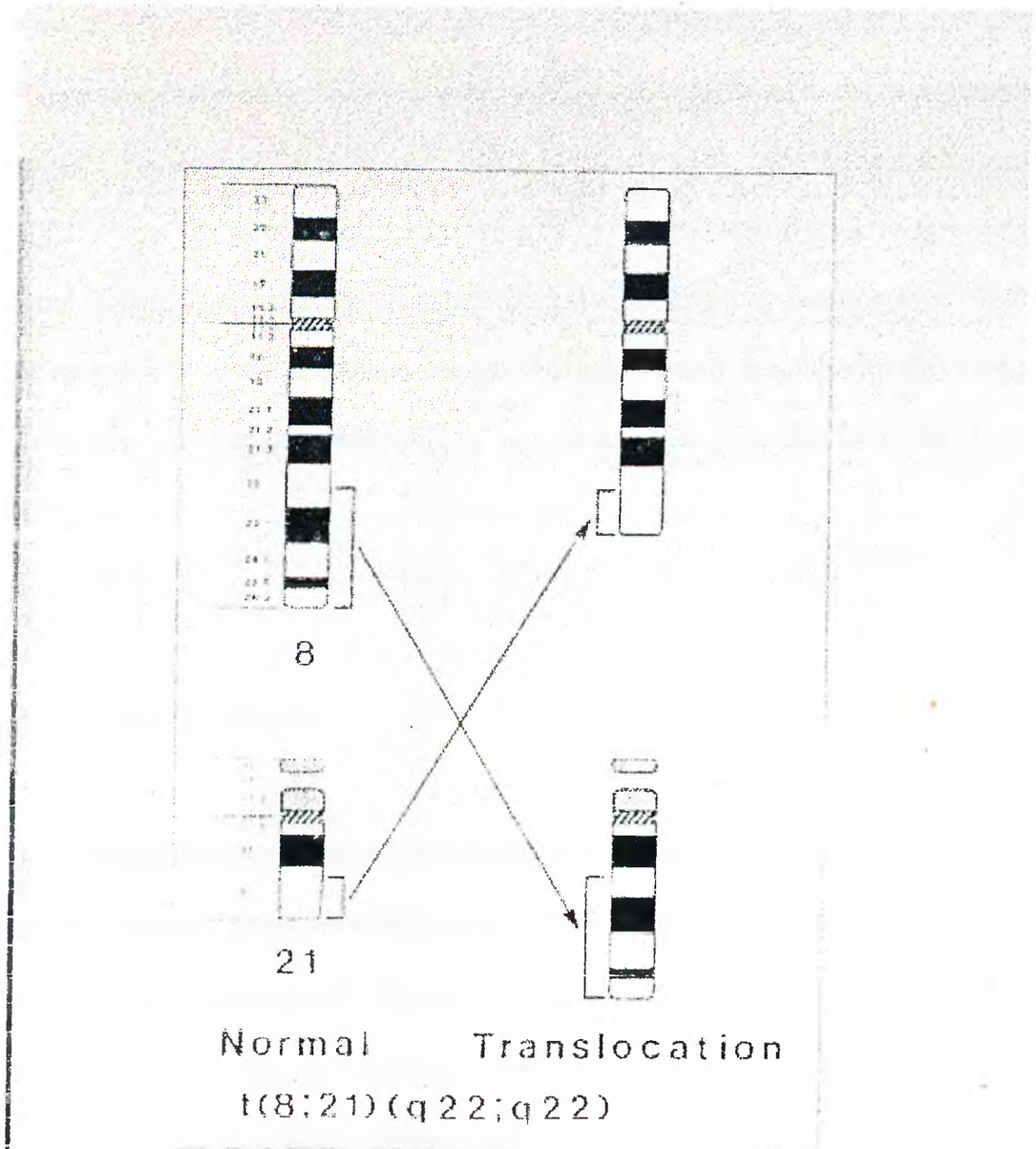

Figure 4: t (8;21) (q22;q22) Rearrangement

(Kurzrock R., and Talpaz M., 1995)

both AML1 and the fusion protein takes place. The elevated levels of *BCL-2* in cells expressing *AML1-ETO* may prolong their life span and contribute to the development of t(8;21) leukemia (Klampfer *et al.*, 1996). In a recent study, mice that mimic human t(8;21) were generated. Mice heterozygous for *AML1-ETO* allele die in midgestation from hemorrhaging in CNS and exhibit severe block in fetal liver hematopoiesis which is very similar to the phenotype obtained by homozygous disruption of the *AML1* gene. This indicates that *AML1-ETO* blocks normal *AML1* function (Yergeau *et al.*, 1997).

1.4.3 t (15;17) (q22;q21)

A consistent translocation t(15;17) is present in the blasts of the majority of the APL patients. The two genes involved are *MYL* (also called *PML*) on chromosome 15, and RAR-α on chromosome 17. Chimeric *RAR-α / MYL* (on the 17q- derivative) and *MYL / RAR-α* (on the 15q+ derivative chromosome) are generated as a consequence of the reciprocal translocation (Figure 5) (Biondi *et al.*, 1992).

Retinoic acid receptors (RARs) are nuclear hormone receptors that act as retinoic acid (RA)-inducible transcriptional activators in their heterodimeric form. RA controls fundamental developmental processes, induces terminal differentiation of myeloid hemotopoietic progenitors, and has tumor and cell growth suppressive activities. The chimeric PML-RAR-α protein contains most of the *PML* sequences fused to a large

part of *RAR-α*, including its DNA and hormone binding domains (Wang *et al.*, 1998).

PML is an interferon (IFN)-inducible gene that encodes a ring-finger protein which is typically concentrated within discrete speckled nuclear structures called PML nuclear bodies or PML oncogenic domains. Ablation of murine Pml protein by homologous recombination revealed that PML regulates hemopoietic differentiation and controls cell growth and tumorigenesis. *PML* function is essential for the tumor growth suppressive activity of RA and for its ability to induce terminal myeloid differentiation of progenitor cells. Thus *PML* is a critical component of the RA pathway and disruption of its activity by the translocation may be important in APL pathogenesis (Wang *et al.*, 1998).

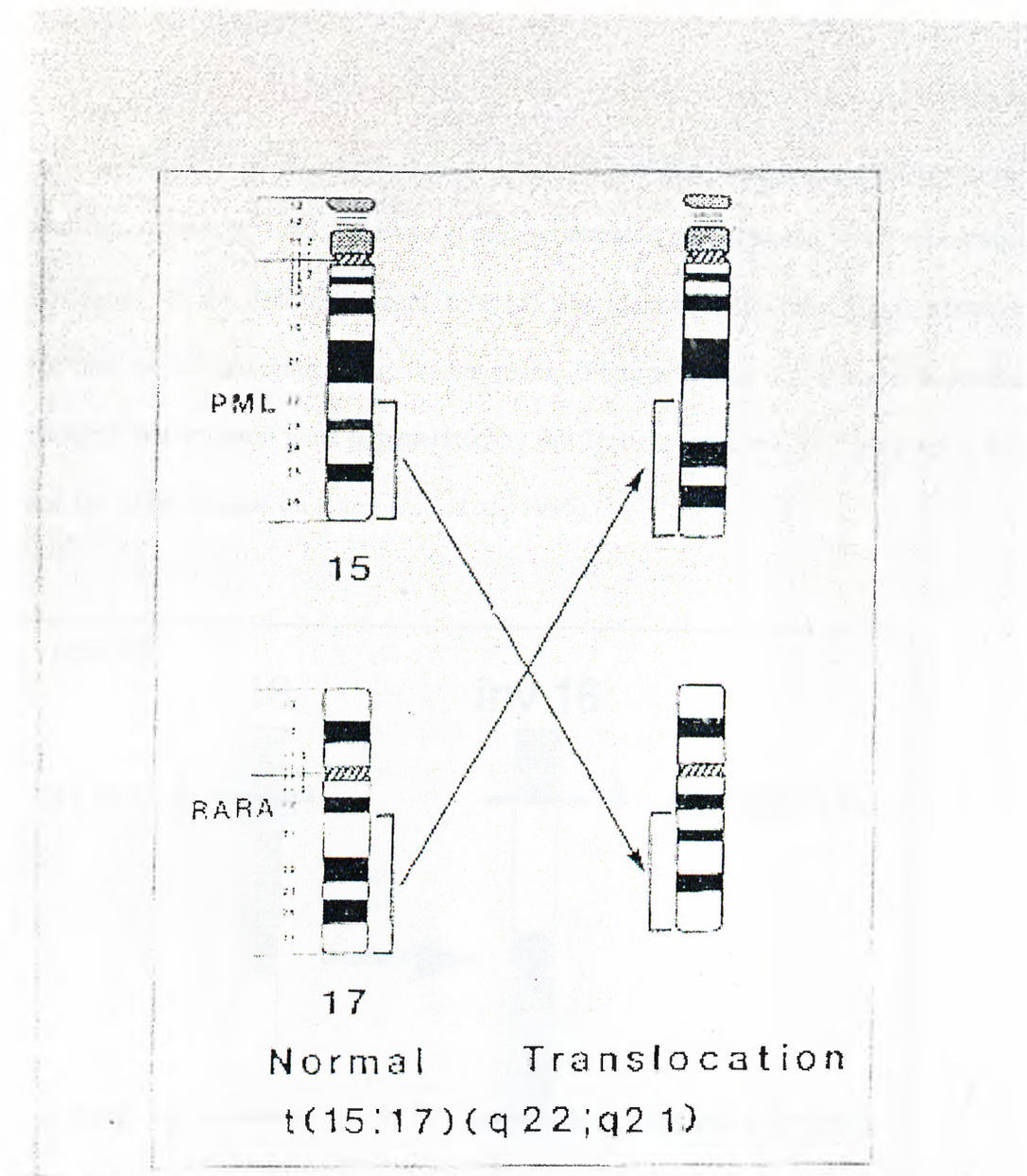

Figure 5: t (15;17) (q22;q21) Rearrangement

(Kurzrock R., and Talpaz M., 1995)

1.4.4 inv 16 (p13;q22)

The involvement of chromosome 16 in leukemia was first reported in 1982 by Arthur and Bloomfield. In 1983, Le Beau *et al* demonstrated chromosome 16 inversion with AML-M4. In the following years, t(16;16) was identified, the breakpoints of which seemed to be the same as in the inversion (Figures 6 and 7). Molecular studies showed that a fusion gene is generated by inv 16 between the *CBFB* gene on q arm and the *MYH11* gene on p arm (Liu *et al.*, 1995).

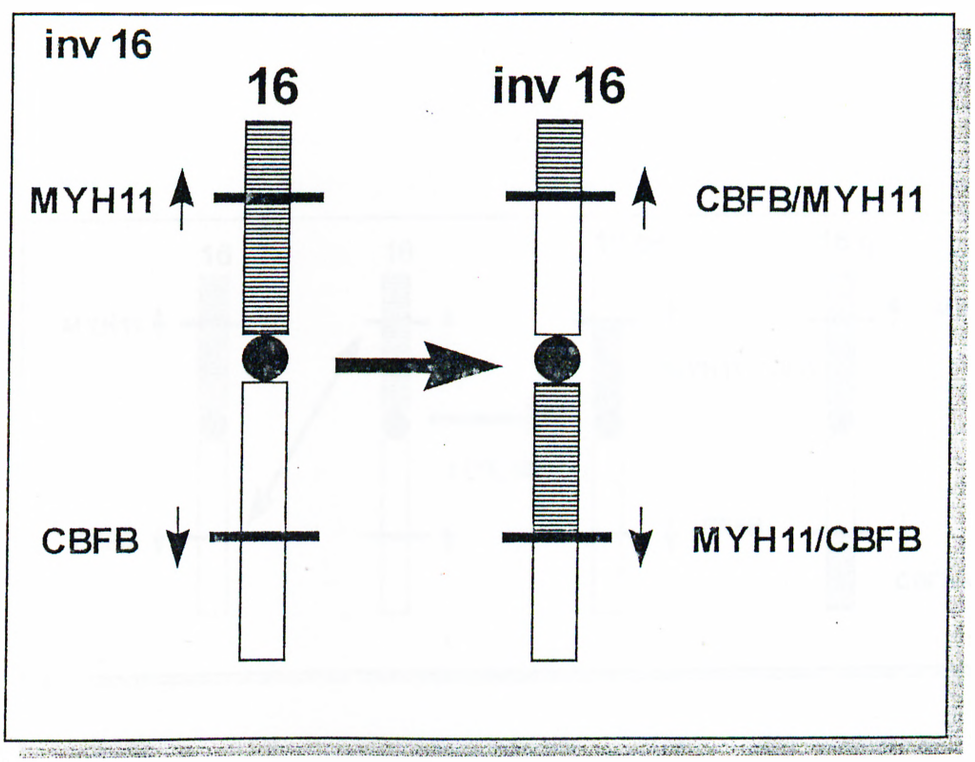

Figure 6: inv 16 (p13;q22) Rearrangement

(Adapted from Claxton *et al.*, 1994)

CBFB gene encodes a subunit of the transcription factor complex CBF, also known as PEBP2. PEBP2 was originally identified as a protein in NIH 3T3 cells that binds to mouse polyoma virus enhancer. CBF was identified as a protein predominantly expressed in T cells that bind to a conserved "core" site in murine leukemia virus enhancers. Both PEBP2 and CBF recognize the same core site. The cloning and purification identified them as the same protein. CBF consists of two subunits: the α subunit, which binds the DNA target, and the β subunit, which does not bind DNA directly. At least three α subunits have been identified one of which is encoded by *AML1* gene that is disrupted by t(8;21) and t(3;21) (Liu *et al.*, 1995).

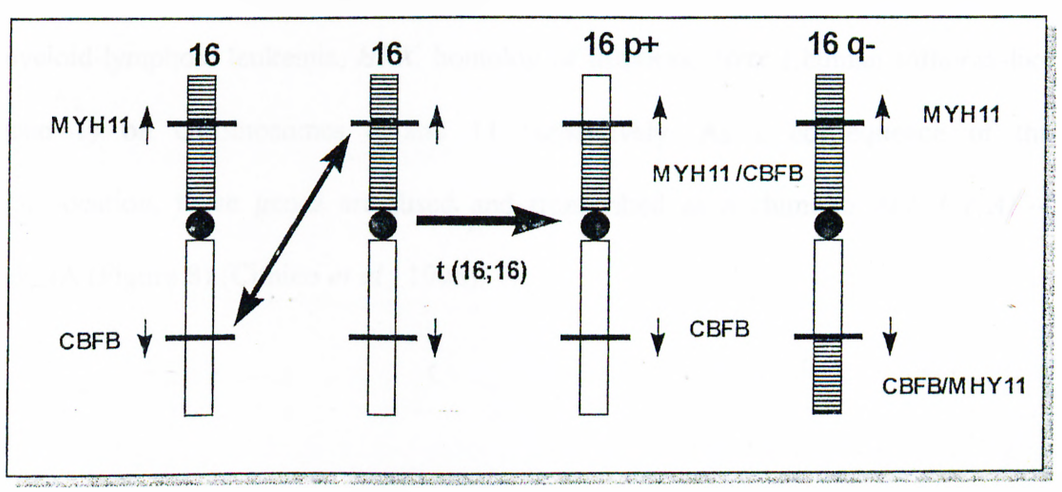

Figure 7: t (16;16) (p13;q22) Rearrangement

(Adapted from Claxton *et al.*, 1994)

The smooth muscle myosin heavy chain (SMMHC) gene *MYH11* is a member of the myosin II family. A typical myosin II protein has an ATPase head, responsible for actin binding and mechanical movement, a hinge region, and a long tail with tandem copies of a coiled-coil domain that facilitates the filament assembly. The breakpoints identified are within the tail region, fusing the C-terminal portion of the tail to *CBFB* (Liu *et al.*, 1995).

1.4.5 t (4;11) (q21;q23)

The t (4;11) (q21;q23) cytogenetic abnormality is associated with a subset of ALL. The two genes involved are *AF-4* (*FEL*) and *ALL-1* (*MLL*, mixed lineage leukemia or myeloid-lymphoid leukemia, *HRX*, homolog of trithorax, *Hrtx 1*, human trithorax-like gene 1) on chromosomes 4 and 11 respectively. As a consequence of the translocation, these genes are fused and transcribed as a chimeric *ALL-1 / AF-4* mRNA (Figure 8) (Cimino *et al.*, 1996).

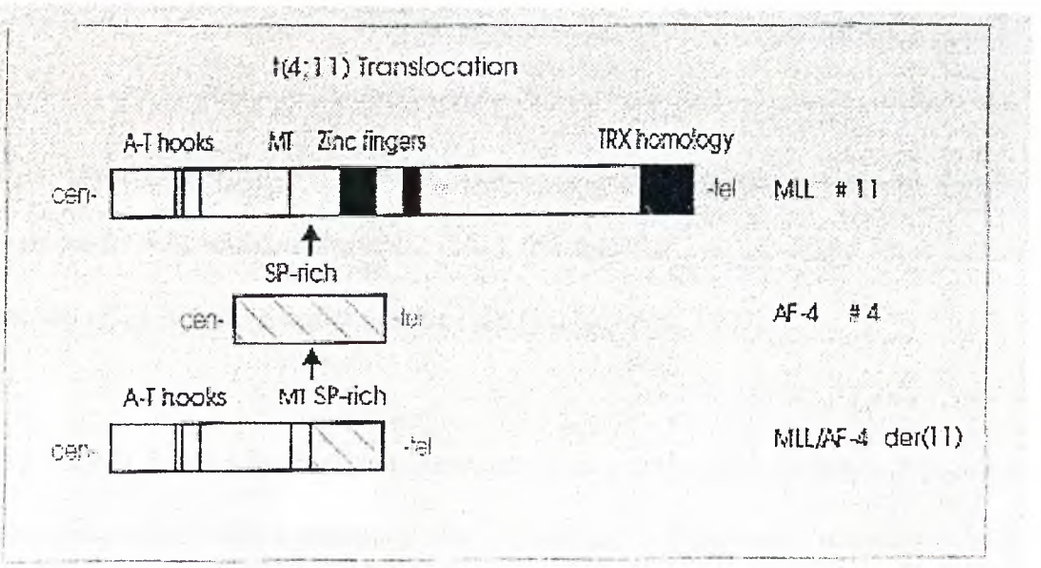

Figure 8: t (4;11) (q21;q23) Rearrangement

(Kurzrock R., and Talpaz M., 1995)

The *ALL-1* gene is found rearranged in approximately 10 % of ALL and in over 5 % of AML. The gene undergoes fusion with different partners on a variety of chromosomes such as chromosomes 1, 4, 6, 9 ,10, or 19. The gene, which spans a region on chromosome band 11q23 approximately 90 kb in length, consists of 36 exons (Rasio *et al.*, 1996). *ALL-1* gene belongs to the trithorax gene family of which the Drosophila trithorax (trx) gene is known to regulate homeotic genes through alternative splicing. Nam *et al.* demonstrated that, different cell types transcribe *ALL-1* mRNA species lacking exons that generally encode putative regulatory domains such as AT hooks (exon3), repression domain (exon 6), zinc finger motifs (exon 8),

and activation domain (exon 18). This suggests that posttranscriptional regulation by alternative splicing may play an important role in *ALL-1* gene expression (Nam *et al.*, 1996). Immunocytochemical analysis showed that the protein localizes to nuclear structures in cells with and without 11q23 translocations. It is widely expressed in most cell types including hematopoietic cells (Butler *et al.*, 1997).

AF-4 (ALL-1 fused gene from chromosome 4), is a serine and proline rich putative transcription factor with a glutamine rich C-terminus. It is expressed normally in both B- and T-lymphoid cell lines . The *ALL-1 / AF-4* fusion protein contains a large C-terminal portion of *AF-4* fused to the N-terminal portion of *ALL-1* containing its AT hook DNA binding motifs (Morrissey *et al.*, 1993).

1.4.6 t (1;19) (q23;p23)

The prognostically important 1;19 chromosomal translocation can alter the *E2A* gene on chromosome 19p13 in childhood B-cell precursor ALL. The breakpoint on chromosome 1q23 interrupts a homeobox gene known as *PBX1*. As a result, chimeric transcripts are formed that retain the activator domain of the *E2A* gene but substitute a homeobox domain of the Pbx1 protein for the helix-loop-helix DNA binding and dimerization domain of *E2A* (Figure 9) (Privitera *et al.*, 1992).

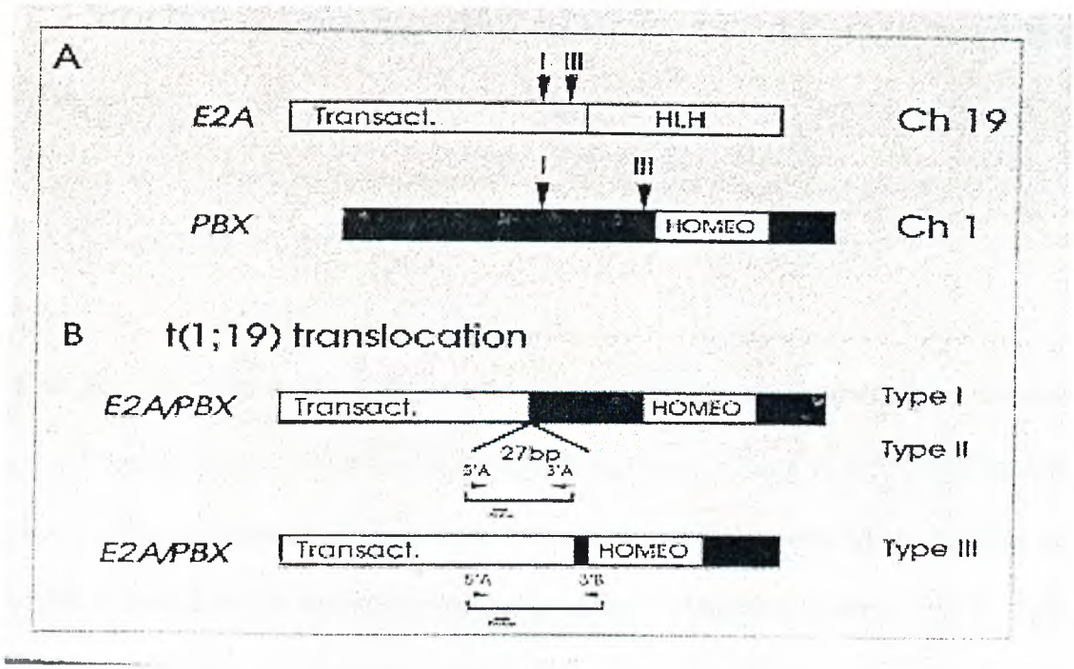

Figure 9: t (1;19) (q23;p23) Rearrangement

(Kurzrock R., and Talpaz M., 1995)

E2A gene encodes transcription factors of the helix-loop-helix (HLH) family that are implicated in cell-specific gene expression as part of dimeric complexes that interact with E box enhancer elements. Its expression is detected in a wide range of cell lines. *E2A* gene gives rise to two proteins, E12 and E47, by differential splicing of E12- and E47- specific basic HLH encoding exons. Both have been implicated in the regulation of Ig gene expression (Aronheim *et al.*, 1993).

I. 2. MOLECULAR DIAGNOSIS OF HEMATOLOGICAL MALIGNANCIES

2.1 Cytogenetics

Cytogenetics is the study of the genetic material, genome or chromosomes, of a cell by cytological means. In molecular cytogenetics, the advantage of the specificity and base pairing qualities of DNA is used to look at specific regions of the genome to which it hybridizes. In hematological malignancies, cytogenetic analysis may be used for: (a) detection of malignant proliferations, (b) classification of hematological neoplasms, (c) characterization of the degree of neoplastic progression, (d) testing for remission (disappearance of signs of cancer), and (e) establishment of the time of relapse (Kurzrock *et al.*, 1995).

The application of banding techniques; especially Q (quinacrine fluorescence) and G (Giemsa) bandings are used for detailed characterization of structural rearrangements within chromosomes (Lee *et al.*, 1993).

Molecular cytogenetics provides a powerful link between molecular genetic analysis and chromosome morphology by allowing to pinpoint structurally aberrant chromosome regions on the molecular level (Lichter *et al.*, 1996).

Fluorescence In Situ Hybridization (FISH)

In situ hybridization is the term used to describe a procedure in which labelled DNA is hybridized to cell or tissue preparations on slides. In molecular cytogenetics, this procedure is used to identify chromosomes or chromosome regions containing the probe sequences (Kurzrock *et al.*, 1995).

The advantages of this technique may be summarized as follows:

a. it allows the study of chromosome changes by scoring large numbers of non-dividing cells in a relatively short time,
b. it enables better definition of the nature of complex structural changes in metaphase cells,
c. it permits estimation of the size of abnormal clones in interphase cells, and
d. it may help in determination of the lineage of cells carrying a chromosome abnormality (Cuneo *et al.*, 1997).

Besides FISH, there are other molecular cytogenetics methods such as comparative genomic hybridazation (CGH), which may prove to be very useful for diagnostic purposes in the coming years.

2.2 Immunological Methods

In addition to cytogenetic analysis, a variety of immunologic techniques have been developed for more specific and accurate diagnosis. Immunofluorescence and immunoenzymatic techniques allow localization of cell or tissue antigens on the surface, in the cytoplasm, or in the nucleus. In the direct immunofluorescence techniques, the antigen is identified by a fluorochrome-labelled specific antibody. The more sensitive indirect method utilizes an unlabelled specific primary antibody and allows it to react with the tissue antigen. A fluorochrome conjugated second antibody (anti-immunoglobulin) that binds to the primary antibody is then applied. Immunofluorescence techniques are used mostly to study cells in suspension by means of fluorescence microscopy or automated flow cytometers. There are also immunoenzymatic tests which utilize immunoperoxidase technique, the avidin-biotin complex system, and the immunoalkaline phosphatase technique (Lee *et al.*, 1993).

2.3 Fusion Transcript Detection

Recurring chromosomal abnormalities are closely associated with particular phenotypes. This is especially the case for structural chromosomal changes seen in leukemias and lymphomas, which have diagnostic and prognostic implications. Molecular analysis of these changes not only yields important insights into disease pathogenesis but also provides more precise definition of disease subsets which is

critical in the management of patients with these rearrangements (Claxton *et al.*, 1994).

The advent of polymerase chain reaction (PCR) technology has greatly facilitated the analysis of small nucleic acid samples. In cases where there is sufficient molecular information, reverse-transcriptase polymerase chain reaction (RT-PCR) is particularly useful. In this approach, the isolated RNA is converted to single-stranded cDNA (complementary DNA), which can then be used for enzymatic amplification of sequences between specific primers. The primers are designed such that amplification will be achieved only if the chromosomal rearrangement under question has taken place. Diagnostic PCR products are then easily detected by hybridization and / or electrophoresis techniques. Several advantages of RT-PCR over other molecular methods can be listed as follows:

a. It is rapid and sensitive, fusion transcipts are amplifiable from cDNA synthesized from as little as 20 pg of total cellular RNA which is equivalent to the 2-5 cell level. Standard Southern gel analysis on the other hand, requires about 5% of cells to be leukemic for the detection of deletions and / or translocations in genomic DNA.
b. PCR amplification of coding sequences in genomic DNA requires knowledge of the exon-intron structure of the gene, because interruptions of the coding sequences with large introns can make amplification difficult or impossible (Kawasaki *et al.*, 1988).

I. 3. TREATMENT OF HEMATOLOGICAL MALIGNANCIES

The accumulation of leukemic blasts in bone marrow suppresses normal hematopoietic stem cells. Therapeutically the aim is to decrease population of leukemic clone enough to allow the recovery of normal stem cells (Cotran *et al.*, 1989).

In order to 'cure' a malignancy, all of the cancer cells must be destroyed. Hormones, cytokines (interferons, interleukins, tumor necrosis factor), monoclonal antibodies coupled to tumoricidal agents, cells (lymphokine activated killer (LAK) cells, tumor infiltrating lymphocytes (TIL)), and chemotherapeutic drugs are among biologic and chemical agents which are used in cancer treatment (Wilson *et al.*, 1991).

3.1 Chemotherapy and Biologic Agents

In order to achieve a cure with chemotherapeutic drugs, (1) the cancer cells must be sensitive to the agent; (2) the drug must reach the malignant cell; (3) if the drug is effective only in a phase of the cell cycle, it must be given frequent enough that all the cancer cells enter this phase of the cycle in the presence of the drug; and (4) the malignant cells must be destroyed before drug resistance emerges (Wilson *et al.*, 1991).

Antineoplastic drugs exert their cytotoxic effects by interfering with different cellular mechanisms involved in cell growth. These include alkylating agents, antineoplastic antibiotics, plant alkaloids, and antimetabolites. *Alkylating agents* have their cytotoxic effects through covalent binding of the alkyl groups to cellular DNA or other molecules. Examples are cyclophosphamide, the chlorethylnitrosoureas, and melphalan. *Antineoplastic antibiotics*, which are isolated from microbial fermentation extracts, are responsible for the improved treatment of leukemias and lymphomas. They include bleomycin, doxorubicin, mitomycin, and mitoxantrone. *Plant alkaloids*, are also effective antineoplastic drugs derived from natural sources; examples are vincristine, vinblastine, etoposide and teniposide. Finally *antimetabolites* act by inhibiting crucial metabolic enzymes. 5-fluorouracil, methotraxate, and cytarabine are mostly effective on rapidly dividing cells (Wilson *et al.*, 1991).

There are different biologic approaches to the treatment of cancer. These agents act through one of the following mechanisms : (1) augmenting the defenses of the host, (2) direct tumoricidal effect, and (3) modification of the behaviour of the tumor. Cytokines and hematopoietic growth factors are frequently used biological agents (Wilson *et al.*, 1991).

Granulocyte (G) or granulocyte-macrophage (GM) colony stimulating factors (CSF), interferon- α (IFN-α), and interleukin 2 (IL-2) are among the widely used therapeutic agents. AML blasts have surface receptors for CSFs and may proliferate in response to CSFs. By this way, leukemic cells are sensitized by CSFs, made more susceptible to

chemotherapy which enables to increase the dose intensity of chemotherapeutic agents. CSFs have also been used to mobilize peripheral blood stem cells for autologous and allogeneic transplantation (Ezaki K., 1996).

Interferons originate from natural sources and are products of recombinant technology. There are three major classes of interferons: interferons alpha, beta, and gamma. Interferons alpha, which have antiviral, anticancer, and immunomodulatory activities, are secreted and synthesized by leucocytes and lymphoblasts. IFN-α therapy is used in the management of patients with hematological diseases, malignancies, lymphomas, solid malignant tumors and viral infections. For CML, it is considered as a first-line therapy option in patients who cannot receive or relapse following allogeneic bone marrow transplantation (Bajcetic *et al.*, 1998).

Vitamin A and its natural and synthetic derivatives, the retinoids are required for many essential life processes, including vision, reproduction, metabolism, differentiation, hematopoiesis, and bone development. There is also evidence that retinoids have a potent antiproliferative effect, and may be effective in the treatment of different human diseases including cancer. Apart from vision, retinoids exert these diverse effects by regulating gene expression at specific target sites within cells. The three retinoids of known biological importance are retinol, retinal, and retinoic acid (Sacchi *et al.*, 1997).

Studies over the last two decades demonstrated the capacity of some substances to induce *in vitro* differentiation of human leukemia cell lines. In 1988, a Chinese group showed that by using all-trans retinoic acid (ATRA) alone, complete remission through differentiation of the leukemic clone was obtained in 94% of acute promyelocytic leukemia (APL) patients. One of the most important features of APL is the t(15;17) (q22;q21) chromosomal translocation leading to the production of two fusion genes, *PML / RAR-α* and *RAR-α / PML*. While the former hybrid is transcribed in all APL patients, the latter is detectable in only 70% of cases. Therefore, detection of PML / RAR-α fusion protein mRNA via RT-PCR technique has proved to be extremely important for molecular diagnosis and monitoring of treatment response in APL patients. Studies have shown that ATRA should be incorporated into the front line of therapy of newly diagnosed APL (Sacchi *et al.*, 1997).

3.2 Transplantation of Hematopoietic Cells

High-dose cytotoxic therapy with stem cell rescue (*ie,* bone marrow transplantation or BMT) is frequently the treatment of choice for many patients with malignancies and nonmalignant diseases that affect blood cells (Jones R. J., 1998).

The main purpose of BMT and peripheral blood stem cell transplantation (PBSCT) procedures in cancer treatment is to enable the application of very high doses of chemotherapy and radiation therapy to increase their effectiveness. BMT and PBSCT

allow stem cells that were damaged by treatment to be replaced with healthy stem cells (http: // www. cancernews. com)

BMT is not only applied for the treatment of leukemias and lymphomas but also for the breast, lung, ovary, germ cell tumors, multiple myeloma, some primary brain tumors, and some head and neck cancers. Several noncancerous disorders such as aplastic anemia, severe combined immunodeficiency disease (SCID), thalassemia, and myelodysplastic syndromes are also being treated with BMT (http: // www. cancernews. com).

There are three groups of BMT. In ***syngeneic transplantation***, bone marrow is taken from an identical twin. ***Allogeneic transplantation***, in which the donor is a person other than the patient or an identical twin, is more common. The success of allogeneic transplantation depends on how closely the HLA (human leukocyte-associated antigens) antigens of donor's marrow match those of the recipient's marrow. Finally, in ***autologous transplantation***, patients receive their own marrow or peripheral stem cells. The type of transplant the patient receives depends on the type of disease and the availability of a suitable donor (http: // www. cancernews. com).

PBSCT is being used more frequently in both the autologous and allogeneic setting. Normally, progenitor cells are present in low quantities in circulating blood. However, stem cells can be recruited or mobilized into the peripheral blood circulation. The advent of strategies for mobilization by the growth factors following subsequent

harvest of stem cells from the peripheral circulation, has resulted in an increased yield of stem cells. Recombinant growth factors such as GM-CSF and G-CSF are commonly used (Repka T., and Weisdorf D., 1998).

The two major problems in allogeneic transplantation are graft-versus-host disease and graft failure.

Leukemia Relapse After Allogeneic Transplantation

After successful allogeneic BMT, hematopoiesis and immunity is reconstituted from donor-derived cells. Leukemia relapse generally occurs in recipient-derived cells. This indicates that clonogenic malignant cells survived the high-dose chemoradiotherapy and avoided the graft versus leukemia (GVL) effect (Giralt S. A., and Champlin R. E., 1994).

Relapse rarely occurs in donor-derived cells. The mechanisms postulated for leukemic transformation of donor cells include radiation-facilitated viral leukemogenesis, persistence of the leukemic stimulus with *de novo* leukemic transformation, or transfer of oncogenic genetic material from host-derived leukemic cells to normal donor hematopoietic cells. Patients with more advanced disease have a higher relapse rate approaching 50 % to 70 % with T-cell depletion (Giralt S. A., and Champlin R. E., 1994).

Treatment of recurrent leukemia after allogeneic BMT has been associated with limited success. The administration of INF-α, IL-2 has been shown to reinduce remission in some cases of relapsed acute leukemia and CML after allogeneic BMT. In cases of relapse later than 12 months, a second transplantation may be associated with 20 % to 30 % long term DFS (disease free survival) (Tabbara I. A., 1996).

In addition, there are experimental treatments that include adoptive immunotherapy, gene therapy, and anti-sense approach.

Bone Marrow Transplantation in Turkey

Bone marrow transplantation centers in Turkey are shown on the following map (Figure 10). Approximately 200 new transplantations are performed per year and nearly half of them are done at Ankara University, School of Medicine, the Department of Hematology. The centers are as follows:

1 a. Ankara University, School of Medicine, İbn-i Sina Hospital

1 b. Ankara University, School of Medicine, the Department of Pediatrics

2 a. Hacettepe University, School of Medicine, İhsan Doğramacı Children's Hospital

2 b. Hacettepe University, School of Medicine, the Department of Hematology

3. Gülhane Medical Academy (GATA) Ankara

4. Erciyes University

5. Çukurova University

6. Akdeniz University

7. Ege University

8. Marmara University

9. Gülhane Medical Academy (GATA) Haydarpaşa

10 a. İstanbul University, İstanbul Faculty of Medicine, the Department of Internal Medicine

10 b. İstanbul University, Cerrahpaşa Faculty of Medicine, the Department of Internal Medicine

11. Bizim Lösemili Çocuklar Vakfi

12. Tepecik Hospital

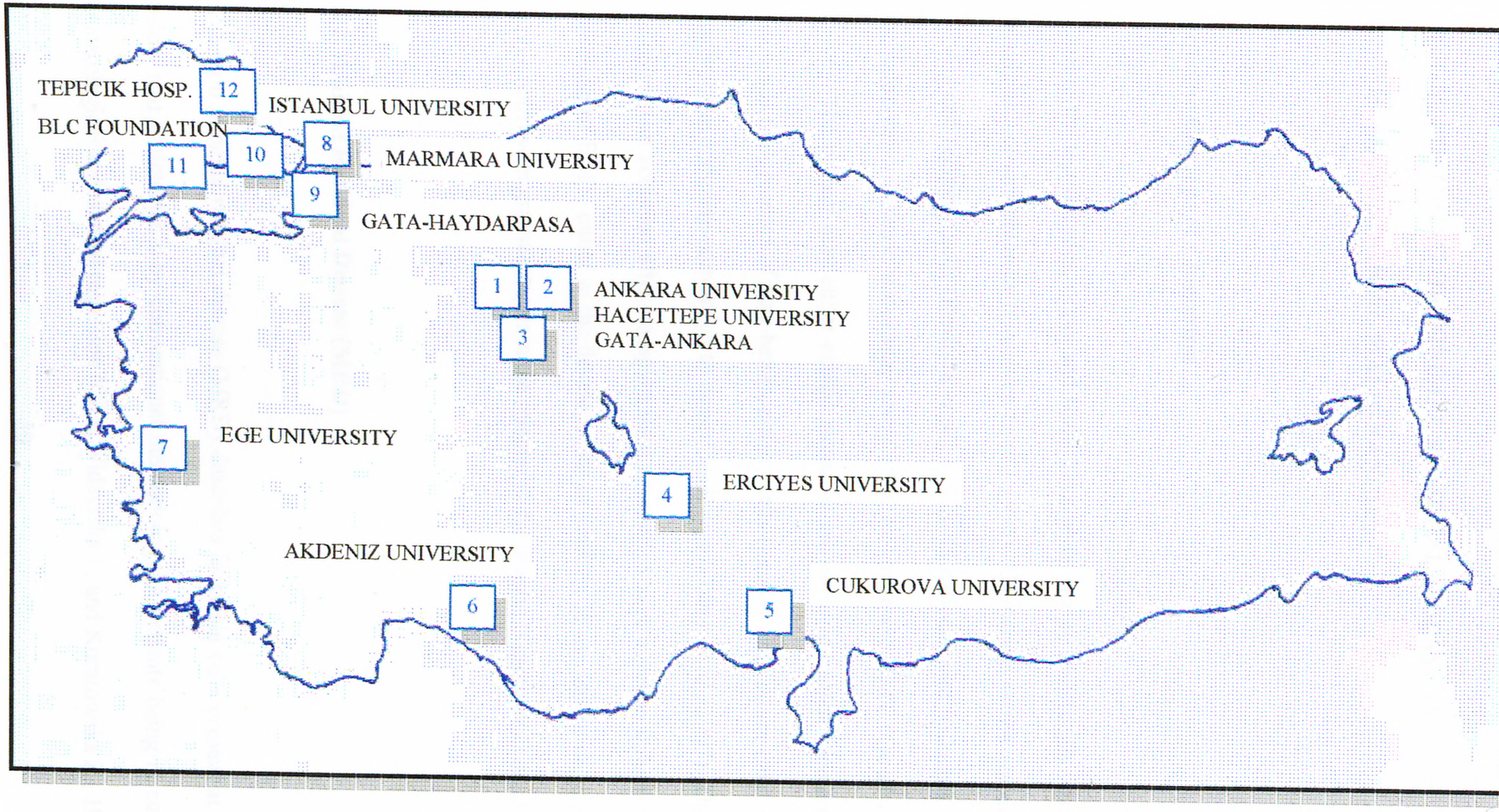

Figure 10: Bone Marrow Transplantation Centers in Turkey

I. 4. MONITORING OF MINIMAL RESIDUAL DISEASE IN HEMATOLOGICAL MALIGNANCIES

4.1 The Concept of Chimerism

During the last decades, organ and BMT have given rise to individuals who are true artificial biologic chimera (Gosalvez *et al.*, 1996). Chimerism is the coexistence of donor and recipient cells (Privitera *et al.*, 1992). In patients with leukemia, different hematopoietic chimeric states may result after an allogeneic BMT (Gosalvez *et al.*, 1996). The patient may either exhibit *mixed chimerism* or *complete chimerism*. The former is the presence of host hematopoietic cells together with donor cells while the latter is the complete conversion to donor's cell type (Ozbek *et al.*, 1997). The degree of chimerism achieved after transplantation appears to have a strong influence on the outcome of BMT, either in the development of GVHD, in the failure or rejection of stable marrow engraftments, or in the relapse of the disease (Gosalvez *et al.*, 1996).

4.2 Minimal Residual Disease (MRD)

The term minimal residual disease (MRD) describes leukemia cells present at a level below that is detectable by conventional methodology in patients being in complete hematological and clinical remission (CR) (Widzysska I., and Kuratowska Z., 1995).

Clinically, MRD can be an indicator for a prediction of relapse. As an example, a greater MRD on entering CR tends to be related with an early relapse, a return to MRD-positive after disappearance of MRD will be a sign of impending relapse, and MRD negativity at the termination of therapy may be correlated with a long-term DFS. Therefore, more precise evaluation of MRD is necessary with regard to therapeutic strategy in monitoring of the disease (Misawa *et al.*, 1995).

4.3 Direct Monitoring of MRD Using Chromosomal Rearrangements

The detection of MRD has gained special significance in BMT due to the possibility not just to detect but also to prevent, treat and reinduce remission in patients that relapsed post-BMT by immunotherapy (Toren *et al.*, 1996). During the last decade, several techniques have been proposed and used for detecting MRD each of which is characterized by advantages and limitations, mainly related to its sensitivity and specificity. The general limitations of such tests originates from the size of the sample that can be analyzed and the heterogeneous distribution of leukemia after treatment (Carlo - Stella *et al.*, 1995).

MRD can be detected by various modern techniques such as cytogenetics, RFLP analysis, analysis of VNTRs by Southern blot or PCR, microsatellite sequences, PCR amplification products of the Y chromosome or the Amelogenin gene, quantitative PCR and FISH (Toren *et al.*, 1996).

The very rapid development of techniques based on use of PCR for characterization of molecular lesions in leukemia and lymphoma also offers the opportunity for monitoring residual disease at a sensitivity of one malignant cell in 10(5) or 10(6) normal cells. Maximal specificity is achieved when the DNA sequences amplified are leukemia specific such as *BCR / ABL* in CML, *PML / RAR-α* in APL, *AML-1 / ETO* in t (8;21) in AML and *CBFB / MYH11* in inv 16 AML. A considerable level of sensitivity may also be achieved by using Ig heavy chain (IGH) and TCR gene rearrangements (Saglio G., 1995).

4.4 Indirect Monitoring of MRD Using DNA Polymorphisms

Several indirect analysis methods have been used to evaluate chimerism following BMT. These include protein polymorphisms, chromosomal studies and DNA typing. PCR has been used for the rapid and sensitive analysis of human DNA variations which are inherited in a Mendelian fashion. These variations include highly polymorphic tandemly repetetive minisatellites and single locus polymorphisms (Ozbek *et al.*, 1997).

Restriction fragment length polymorphisms (RFLPs) are DNA variants that are caused by *single nucleotide polymorphisms* (SNPs). Another common class of RFLP is due to variable number of tandem repeats (VNTR) resulting in the restriction fragment length variability. These are called *minisatellites* and show extensive size

variation with many alleles per locus. *Microsatellites* or dinucleotide repeats were identified as a subclass of VNTRs. They show a high level of length variation and are distributed approximately randomly throughout the genome (Schafer *et al.*, 1997).

Short tandem repeat (STR) loci consist of simple tandemly repeated sequences of 1-6 bp in length. STRs may exhibit a high degree of length polymorphism due to the variable number of repeat units. They appear to be abundant throughout the human genome and occur, on average, every 6-10 kb. Polymorphic STR sequences are present in both intragenic and extragenic regions. Their abundance, polymorphic nature, and availability for amplification by PCR, make STRs ideal genetic markers. In addition, DNA profiling based on PCR amplification of STRs has the advantage of being more sensitive than conventional techniques (Kimpton *et al.*, 1993).

RFLPs are useful genetic markers for documentation of hematopoietic chimerism after BMT as well as for determining the origin of leukemic cells in BMT patients with recurrent disease. Amplification of multiple polymorphisms and their analysis provides a way to perform rapid genotyping. These amplified sequence polymorphisms are ideal for chimerism evaluation after BMT between sibling pairs because several polymorphic loci must be analyzed to achieve a high probability of having at least one informative locus. The major advantage of using PCR-based analysis for this purpose is increased sensitivity, thereby making it possible to detect a small proportion of DNA of donor or recipient origin. In addition, as the quality of the DNA used in the analysis is less critical, abbreviated methods may be used for DNA

preparation; only small amounts of DNA are required, and the analysis is more rapid than traditional RFLP analysis (Ugozzoli *et al.*, 1991).

The detection of a complete chimerism is a good prognostic factor in patients who undergo BMT. Although, a complete conversion to donor hematopoiesis is expected following allogeneic BMT, it is known that persistence or reappearance of recipient hematopoiesis is not an unusual event. These recipient cells can either be malignant or non-malignant. The reappearance of malignant host cells indicates relapse, while normal recipient cells may coexist with donor cells for various periods of time resulting in situations called transient or stable mixed hematopoietic chimerism (Suttorp *et al.*, 1993).

In this study four STR loci of tetranucleotide repeats have been used (Table 8). In addition to the four STR loci described, a PKU (phenylketonuria) VNTR polymorphism in PAH gene was also studied.

Table 8 : Locus Specific Information of the STR Loci Studied

STR Locus	GenBank Locus and Locus Definition	Chromosomal Location	Repeat Sequence 5' - 3'	Size Range (bases)
vWA (vWF)	**HUMVWFA31**	12p12 - pter	AGAT	139 - 167
THO1	**HUMTHO1**	11p15 - 15.5	AATG	179 - 203
F13A01	**HUMF13A01**	6p24 - 25	AAAG	283 - 331
FESFPS	**HUMFESFPS**	15q25 - qter	AAAT	222 - 250

(Adapted from Gene Print™ STR Systems Promega Technical Manual)

Regarding Table 8 :

HUMVWFA31 : Human von Willebrand factor gene

HUMTHO1 : Human tyrosine hydroxylase gene

HUMF13A01 : Human coagulation factor XIII a subunit gene

HUMFESFPS : Human c-fes / fps proto-oncogene

I. 5. AIM

The aim of this thesis is the development of PCR-based tests for the diagnosis and also monitoring of patients with different hematological malignancies. In this context, we focused on two aspects: The first one is DNA polymorphism based evaluation of chimerism status of patients following allogeneic transplantation. The second aspect is reverse-transcriptase polymerase chain reaction (RT-PCR) based analysis of breakpoints generated by chromosomal rearrangements in various acute and chronic leukemias.

As research advances, the general transplantation procedures for the treatment of hematological malignancies are modified. On the way to obtain the maximum survival rate with minimum post-operation complications, molecular studies such as the evaluation of chimerism status is of great value for the establishment of new therapeutic strategies. In addition, the repetitive analyses performed at different time intervals in post-transplantation period, is a supportive prognostic approach.

This study is also expected to provide information on the correlation of certain chromosomal rearrangements with the development of acute and chronic leukemias in the Turkish population. Besides, it will be the starting point for determination of exact breakpoints that result in the generation of fusion transcripts.

II. MATERIALS AND METHODS

1. MATERIALS

1.1 Patient Samples

Peripheral blood, bone marrow, and buccal wash samples were obtained from patients with various hematological malignancies in collaboration with Ankara University, School of Medicine, İbn-i Sina Hospital, Bone Marrow Transplantation Unit, and Hacettepe University, İhsan Doğramacı Children's Hospital. Either evaluation of chimerism status after allogeneic transplantation or fusion transcript detection was done using DNA or RNA analyses. Detailed information on these patients is as follows:

Chimerism Analysis

A total of 22 recipient-donor pairs who underwent allogeneic PBSCT for different hematological diseases were evaluated for the chimerism status. The distribution of the patients according to their diagnoses are as follows : Chronic myelogenous leukemia (CML) (n=6), acute myelogenous leukemia (AML) (n=13), β-thalassemia (β-TM) (n=2), and non Hodgkin's lymphoma (NHL) (n=1). Except for one of the β-thalassemia cases (KIT-15), which was from Hacettepe University, all the patients

were referred from Ankara University. Among the AML patients, two of them had M6 (KIT-5, KIT-11), one had M2 (KIT-14) and one had M3 (KIT-22) subtypes. One of the patients (KIT-23) had MDS together with AML. Patient data, disease type, and sex, are summarized in Table 9.

Fusion Transcript Analysis

For the analysis of fusion transcripts, 44 patients diagnosed with acute or chronic leukemias were analyzed. 20 of the patients were female and 24 of them were male. 17 patients were newly diagnosed, and the others underwent allogeneic BMT or PBSCT prior to molecular tests. There are five relapse cases (FS-2, FS-3, FS-7, FS-9, and FS-15), three CML patients are under IFN treatment (FS-17, FS-20, and FS-43), and one patient (FS-47) is under chemotherapy. Of the 44 patients, the diagnoses are as follows: Chronic myelogenous leukemia (CML) (n=26), acute myelogenous leukemia (AML) (n=14), myelodysplastic syndromes (MDS) (n=3), and acute lymphoblastic leukemia (ALL) (n=1). The FAB subtype diagnoses of AML patients are: M1 (FS-8, FS-29, and FS-46), M2 (FS-7, and FS-15), M3 (FS-1, and FS-26), and M4 (FS-2, FS-11, FS-21, and FS-23). One of the AML patients was diagnosed to have biphenotypic AML (FS-14) and the subtypes of the remaining two patients (FS-18, FS-47) are unknown. One AML patient (FS-18) has been in long remission after the treatment. The FAB subtype diagnoses of two MDS patients are RAEB-t (FS-5), and RAEB (FS-6), while that of the third patient (FS-12) is not known. Patient information such as diagnosis, treatment status, and sex are outlined in Table 10.

Table 9 : Patient Samples for Evaluation of Chimerism Status After Allogeneic Transplantation

Case	Disease	Sex (D / R)
1. KIT-1	CML (Ph+)	F / M
2. KIT-4	AML	F / F
3. KIT-5	AML-M6	M / M
4. KIT-6	AML	F / M
5. KIT-10	AML	F / F
6. KIT-11	AML-M6	M / M
7. KIT-12	AML	F / M
8. KIT-13	CML	M / M
9. KIT-14	AML-M2	M / M
10.KIT-15	β-TM	F / F
11.KIT-16	β-TM	M / M
12.KIT-17	NHL	F / M
13.KIT-18	CML	F / F
14.KIT-19	AML	M / M
15.KIT-21	AML	F / M
16.KIT-22	AML-M3	F / M
17.KIT-23	MDS + AML	M / M
18.KIT-24	AML	M / F
19.KIT-25	AML	M / F
20.KIT-29	CML	F / M
21.KIT-30	CML	M / F
22.KIT-32	CML	F / M

D / R: Donor / Recipient
F: Female
M: Male
β-TM: β-thalassemia

CML: Chronic myelogenous leukemia
AML: Acute myelogenous leukemia
MDS: Myelodysplastic syndromes
NHL: Non Hodgkin's lymphoma

Table 10 : Patient Samples for Detection of Fusion Transcripts

Patient Code	Sex	Diagnosis	Treatment
1. FS-1	F	AML-Eo M3	Radiotherapy
2. FS-2	F	AML-M4	Relapse
3. FS-3	M	ALL	Relapse
4. FS-4	M	CML	
5. FS-5	M	MDS-RAEBt	
6. FS-6	M	MDS-RAEB	New diagnosis
7. FS-7	M	AML-M2	1994 BMT, Relapse
8. FS-8	F	AML-M1	New diagnosis
9. FS-9	M	CML relapse	1992 BMT, 1998 Leukocyte infusion, Relapse
10. FS-10	M	Juvenile CML	New diagnosis
11. FS-11	F	AML-M4	New diagnosis
12. FS-12	M	MDS	
13. FS-13	M	CML	
14. FS-14	M	AML Biphenotypic	New diagnosis
15. FS-15	M	AML-M2	Relapse
16. FS-16	F	CML	New diagnosis
17. FS-17	F	CML	Interferon
18. FS-18	M	AML	Long term remission
19. FS-20	F	CML	Interferon
20. FS-21	F	AML-Eo M4	New diagnosis
21. FS-22	F	CML	1997 PBSCT
22. FS-23	F	AML-M4	New diagnosis
23. FS-24	M	CML	
24. FS-25	F	CML	1994 PBSCT
25. FS-26	M	AML-M3	New diagnosis
26. FS-27	F	CML	New diagnosis
27. FS-28	F	CML	1996 PBSCT
28. FS-29	M	AML-M1	New diagnosis
29. FS-30	F	CML	
30. FS-31	M	CML	New diagnosis
31. FS-32	M	CML	PBSCT
32. FS-33	F	CML	New diagnosis
33. FS-34	F	CML	PBSCT
34. FS-35	M	CML	New diagnosis
35. FS-36	F	CML	
36. FS-38	F	CML	New diagnosis
37. FS-39	M	CML	1996 PBSCT
38. FS-40	M	CML	
39. FS-42	F	CML	
40. FS-43	M	CML	Interferon
41. FS-44	M	CML	1997 PBSCT
42. FS-45	M	CML	New diagnosis
43. FS-46	F	AML-M1	New diagnosis
44. FS-47	M	AML	Chemotherapy

CML: Chronic myelogenous leukemia
AML: Acute myelogenous leukemia
PBSCT: Peripheral blood stem cell transplantation
ALL: Acute lymphoblastic leukemia
MDS: Myelodysplastic syndromes
BMT: Bone marrow transplantation

1.2 Chemicals

The list of the chemicals that were used in this study is shown in Table 11:

Table 11 : List of the Chemicals Used in This Study

ITEM	SUPPLIER	CATALOGUE NO
Absolute alcohol	Kimetsan	Kimetsan
Acetic acid (glacial)	Sigma	A6283
Acrylamide	Sigma	A9099
Agarose	Sigma	A9539
Ammonium chloride	Sigma	A9434
Ammonium persulphate (APS)	Sigma	A9164
Boric acid	Sigma	B0252
Bromophenol blue	Sigma	B5525
Chloroform	Sigma	C2432
Diethylpyrocarbonate (DEPC)	Sigma	D5758
EDTA (disodium salt)	Sigma	E5134
Ethanol, absolute	Delta Bios	JDE1
Ficoll Separating Solution	Biochrom KG	L6113
Formaldehyde	Sigma	F8775
Guanidine thiocyanate	Sigma	G6639
Glycerol	Carlo Erba	453751
Hydrogen peroxide	Sigma	M-8600
Isoamylalcohol	Sigma	I9392
Isopropanol	Sigma	I9516
Magnesium chloride	Sigma	M1028
Mercaptoethanol	Sigma	M3158
Metaphor agarose	FMC	50182
MOPS	Sigma	M8899
N-Laurylsarcosine	Sigma	L9150
N-N methylene-bis-acrylamide	Sigma	M2022
Formamide	Sigma	F7508
Phenol	Sigma	P1037
Potassium chloride	Sigma	P4504
Potassium bicarbonate	Carlo Erba	359327
Potassium phosphate (monobasic)	Sigma	P5379
Lauryl sulphate (SDS)	Sigma	L5750
Silver nitrate	Sigma	S8157
Sodium acetate	Sigma	S2889
Sodium chloride	Sigma	S3014
Sodium citrate (trisodium)	Sigma	S4641
Sodium hydroxide	Sigma	S0899
Sodium phosphate (dibasic)	Sigma	S3264
TEMED	Sigma	T8133
Tri Reagent	Sigma	T9424
Tris base	Stratagene	300174
Urea	Sigma	U5378
Xylene cyanol	Sigma	SX4126

1.3 Equipment

The following equipment were used in this study :

1. Aerosol resistant filter (100ul, 200ul, 1000ul), US patent #5364595
2. Balance (Shimadzu Libror EB-2200HU, Shimadzu Libror AEG-120)
3. Black and White Monitor (SSM-121CE)
4. Centrifuge (Beckman GS-15R)
5. E-C Electrophoretic Gel System (MiniCell EC 370M, MidiCell EC 350M, MaxiCell EC 360M)
6. Fluoroscope (Vilber Lourmat)
7. Magnetic stirrer (Thermolyne)
8. Microfuge (Heraeus Biofuge)
9. Millipore Milli-Q Plus PF Ultra-Pure Water System
10. Microwave oven
11. Oligo Synthesizer (Beckman Oligo 1000M DNA Synthesizer)
12. PAGE Apparatus (Bio-Rad Protean® II xi Cell)
13. pH Meter (Consort P501)
14. Pipette Set :P20, P200, P1000 (Eppendorf Research)
15. Plasticware (Pipette tips, eppendorfs, 15 ml and 50 ml falcon tubes) (Costar)
16. Power supply (Bio-Rad Power PAC 200, EC 250-90)
17. Shaker (Thermolyne Roto Mix Type 50800)
18. Sony, UPP-110 Paper
19. Spectrophotometer (Beckman DU 640)

20. Stuart Scientific test tube heater SHT1

21. Thermal Cycler (Perkin Elmer 9600, MJ Research PTC-200)

22. UV Transilluminator (Herolab)

23. Video graphic printer (Sony, UP-890CE)

24. Vortex (Thermolyne Type 16700 Mixer)

25. Water Bath (nüve BM 402)

1.4 Others

Taq Polymerase

The enzyme *Thermus aquatus* DNA Polymerase (native without BSA) was from MBI Fermentas (EP 0282) and was supplied with 10x PCR Buffer and $MgCl_2$ Solution. dNTP Mix (10mM solution) was also from MBI Fermentas (R 0192).

First Strand cDNA Synthesis Kit

The kit used for the synthesis of first strand of cDNA was purchased from MBI Fermentas (K1612).

Components of the kit are as follows :

MuLV-RT : Moloney murine leukemia virus reverse transcriptase

RNAsin : Ribonuclease inhibitor

5x Reaction Buffer

10mM dNTP Mix

Oligo (dT) Primer

Random Hexamer Primer

Control RNA

Control Primer

Deionised water, nuclease free

Proteinase K

Boehringer Mannheim, (1529307) enzyme was used.

Size Markers

1. *pGEM Marker* (Promega, G1741)

The pGEM marker consists of 15 fragments of the following sizes in base pairs : 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, 36.

2. 100 bp DNA Ladder (MBI Fermentas, SM0241)

The marker yields the following 11 discrete fragments in base pairs : 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80.

3. 1 kb DNA Ladder (MBI Fermentas, SM0311)

The marker yields the following 14 discrete fragments in base pairs : 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250.

4. φX 174 DNA / HinfI Marker (MBI Fermentas, SM0261)

HinfI digest of φX 174 DNA yields the following 21 discrete fragments in base pairs : 726, 713, 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118, 100, 82, 66, 66, 48, 42, 40, 24.

1.5 Oligonucleotides

The primers used in this study were all synthesized in the Beckman Oligo 1000M DNA Synthesizer in house, except for the PAH-VNTR primers which were kindly provided by Professor Meral Özgüç from Hacettepe University. The sequences and the other related information are summarized in Table 12.

Table 12 : Primers Used in This Study

Primer Code	Gene	Sequence (5' - 3')	Reference
TC-22 F	HUMVWFA31	CCCTAGTGGATGATAAGAATAATC	Kimpton *et al.*
TC-23 R	HUMVWFA31	GGACAGATGATAAATACATAGGATGGATGG	Kimpton *et al.*
TC-24 F	HUMTHO1	GTGGGCTGAAAAGCTCCCGATTAT	Kimpton *et al.*
TC-25 R	HUMTHO1	GTGATTCCCATTGGCCTGTTCCTC	Kimpton *et al.*
TC-26 F	HUMF13AO1	ATGCCATGCAGATTAGAAA	Kimpton *et al.*
TC-27 R	HUMF13AO1	GAGGTTGCACTCCAGCCTTT	Kimpton *et al.*
TC-28 F	HUMFES/FPS	GGGATTTCCCTATGGATTGG	Kimpton *et al.*
TC-29 R	HUMFES/FPS	GCGAAAGAATGAGACTACAT	Kimpton *et al.*
TC-66 F	β-ACTIN	TGATATCGCCGCGCTC	-
TC-67 R	β-ACTIN	CAGTCAGGTCCCGGCC	-
GA-111 R	C-ABL	TGTGATTATAGCCTAAGACCCGGA	Frenoy *et al.*
GA-112 R	C-ABL	TCCACTGGCCACAAAATCATACAGT	Frenoy *et al.*
GA-431 F	C-ABL	TTCAGCGGCCAGTAGCATCT	Frenoy *et al.*
GA-114 F	BCR	ATGGCGAGGGCGCCTTCCAT	Frenoy *et al.*
GA-113 F	BCR	GTGAAACTCCAGACTGTCCACAGCCA	Frenoy *et al.*
GA-117 F	β-ACTIN	CCTTCCTGGGCATGGAGTCCTG	Biondi *et at.*
GA-118 R	β-ACTIN	GGAGCAATGATCTTGATCTTC	Biondi *et at.*
GA-119 F	MYL	AGCTGCTGGAGGCTGTGGACGCGCCGGTACC	Biondi *et at.*
GA-120 F	MYL	AGTGTACGCCTTCTCCATCA	Biondi *et at.*
GA-121 R	RAR-α	CCACTAGTGGTAGCCTGAGGACT	Biondi *et at.*
GA-122 R	RAR-α	CAGAACTGCTGCTCTGGGTCTCAAT	Biondi *et at.*
GA-123 F	CBFB	GATGCCGCGCGTCGTCCCGGACCA	Claxton *et al.*
GA-124 F	CBFB	AAGCGGCCGCTGAGGGCGGGAAGATGC	Claxton *et al.*
GA-128 F	CBFB	CAGGCAAGGTATATTTGAAGG	Claxton *et al.*
GA-125 R	MYH11	CTCCTCTTCTCCTCATTCTGCTC	Claxton *et al.*
GA-126 R	MYH11	GTCTGTGTTATCTGGAAAGGCTG	Claxton *et al.*
GA-127 R	MYH11	CGTACTGCTGGGTGAGGTTCT	Claxton *et al.*
GA-129 F	E2A	TGCACAACCACGCGGCCCTC	Privitera *et al.*
GA-131 R	E2A	CTTCTCCTCCTCCGAGTGGT	Privitera *et al.*
GA-130 R	PBX1	CGCCACGCCTTCCCCTAACA	Privitera *et al.*
GA-132 F	ALL-1	CGCCCAAGTATCCCTGTAAAAC	Cimino *et al.*
GA-133 R	ALL-1	CTTAAAGTCCACTCTGATCCT	Cimino *et al.*
GA-134 F	ALL-1	GAGGATCCTGCCCCAAAGAAAAG	Cimino *et al.*
GA-135 R	AF-4	TGAGCTGAAGCTGGTCTTCGAGC	Cimino *et al.*
GA-136 F	AML1	AGCCATGAAGAACCAGG	Downing *et al.*
GA-138 F	AML1	TACCACAGAGCCATCAAA	Downing *et al.*
GA-137 R	ETO	AGGCTGTAGGAGAATGG	Downing *et al.*
GA-139 R	ETO	GTTGTCGGTGTAAATGAA	Downing *et al.*
PAH F	PAH	GCTTGAAACTTGAAAGTTGC	Goltsov *et al.*
PAH R	PAH	GGAAACTTAAGAATCCCATC	Goltsov *et al.*

F : Forward primer **R :** Reverse primer

1.6 Solutions

1.6.1 General Stock Solutions

40 % Acrylamide (1L)

380 g acrylamide

20 g N,N-methylenebisacrylamide

is dissolved in 600 ml ddH_2O and the volume is completed to 1L.

0.5 M EDTA (pH: 8.0) (1L)

186.1 g disodium ethylenediaminetetraacetate.2H_2O is dissolved in 800 ml H_2O.

pH is adjusted to 8.0 with NaOH (20 g pellets), and the volume is completed to 1L.

(Sambrook *et al.* Vol 3: B.9-B.14)

1.6.2 Buffers

Phosphate-buffered saline (PBS) (10x, 1L)

80g NaCl

2 g KCl

11.5 g $Na_2HPO_4.7H_2O$

2 g KH_2PO_4

20x SSC

3M NaCl (175g/L)

0.3 M Na_3citrate.$2H_2O$ (88g/L)

pH is adjusted to 7.0 with 1M HCl

TBE Electrophoresis Buffer (10x, 1L)

108 g Tris base

55 g boric acid

40 ml 0.5M EDTA, pH:8.0

TE Buffer (pH: 8.0) (10x, 1L)

10mM Tris.Cl, pH: 8.0

1mM EDTA, pH: 8.0

(Ausubel *et al.* Vol 2: Appendix 2, A.2.3)

1.6.3 DNA Isolation Solutions

10 % SDS

10 g sodium dodecyl sulphate is dissolved in ddH_2O and the volume is completed to 100 ml.

Tris HCl (0.5M, pH: 8.0)

3.94 g TrisHCl is dissolved in 40 ml dd H_2O, pH is adjusted to 8.0, and the volume is completed to 50 ml.

Phenol : Chloroform : Isoamylalcohol (25 : 24 : 1)

25 ml phenol

24 ml chloroform

1 ml isoamylalcohol

Extraction Buffer

10 mM Tris HCl pH: 8.0

10 mM EDTA pH: 8.0

0.5 % SDS

Sodium Acetate (3M, pH: 5.2)

40.8 g sodium acetate is dissolved in 80 ml ddH_2O, pH is adjusted to 5.2 with glacial acetic acid and the volume is completed to 100 ml.

WBCL (White Blood Cell Lysis) Buffer

131mM NH_4Cl

0.9 mM NH_4HCO_4

pH: 6.5

CVS (Chorionic Villus Sampling) Buffer

For 10 ml : 0.01 g SDS

1.5 ml 1M NaCl

0.5 ml 0.5 M EDTA

500 µl Proteinase K (from 20 ng/µl stock)

The volume is completed to 10 ml with ddH_2O.

5 M NaCl

292 g NaCl in 1L ddH_2O.

Isoamylalcohol / chloroform (1 : 24)

1 ml isoamylalcool

24 ml chloroform

1.6.4 Agarose Gel Electrophoresis Solutions

6x Loading Buffer

0.25 % bromophenol blue

0.25 % xylene cyanol FF

30 % glycerol in water

5x Formaldehyde - Gel Running Buffer (1L)

20.6 g MOPS is dissolved in 800 ml DEPC-treated 50 mM sodium acetate and the pH is adjusted to 7.0 with 2N NaOH. 10 ml of DEPC-treated 0.5M EDTA (pH: 8.0) is added. The volume is adjusted to 1L with DEPC-ddH_2O. The buffer yellows with age, if it is exposed to light or is autoclaved.

Formaldehyde - Gel Loading Buffer

50 % glycerol

1 mM EDTA (pH: 8.0)

0.25 % bromophenol blue

0.26 0.25 % xylene cyanol FF

0.1M Ammonium acetate (For staining of the gel)

7.7 g ammonium acetate in 1 L DEPC-ddH_2O

(Sambrook *et al.* Vol 1: 7.43 - 7.45).

1.6.5 Polyacrylamide Gel Electrophoresis Solutions

12 % Denaturing Gel Solution

15 ml 40 % acrylamide solution, 21 gr urea and

5 ml 10x TBE are dissolved and the volume is completed to 50 ml with ddH_2O.

150 µl 10 % APS and 50 µl TEMED are then added.

2x Loading Buffer

950 µl formamide

40 µl 0.5 M EDTA

6 µl ddH_2O

2 µl 2.5 % bromophenol blue

2 µl 2.5 % xylene cyanol

1.6.6 Silver Staining Solutions

Fixative Solution

10 % ethanol and 0.5 % acetic acid (glacial)

Staining Solution

0.1 % AgNO3

Developer Solution

0.1 % formaldehyde

1.5 % NaOH

1.6.7 RNA Isolation Solutions

Solution D

4M Guanidium thiocyanate

25 mM Sodium citrate (pH: 7.0)

0.5 % Sarcosyl

0.1M mercaptoethanol

This solution can be stored at room temperature for 3 months without mercaptoethanol. The addition of this reagent drops the shelf life to 1 month.

DEPC - ddH_2O

1 ml DEPC is dissolved in 1L ddH_2O, the solution is stirred in the hood overnight and then is autoclaved.

2. METHODS

2.1 DNA Isolation From Whole Blood

Blood sample is aliquoted and is frozen in 700 µl aliquots. Prior to the isolation procedure, it is thawn, 800 µl 1x SSC is added ,mixed and spinned in a microfuge for 1 minute. 1.4 ml supernatant is removed and discarded. Following the addition of 1.4 ml 1x SSC, it is briefly vortexed and centrifuged for 1 minute after which all the supernatant is discarded avoiding the pellet. 800 µl Extraction Buffer and 10 µl Proteinase K (20 mg/µl) are then added. After a brief vortex, the mixture is incubated overnight at 56°C.The next day, in a fume hood, 400 µl phenol / chloroform / isoamylalcohol (25: 24: 1) solution is added and vortexed for 1 minute. After spinning for 5 minutes in a microfuge, the upper aqueous layer (~ 700 µl) is removed and placed in a new tube. This step is repeated with 350 µl phenol / chloroform / isoamylalcohol solution until a clear interphase is obtained. The upper aqueous layer containing DNA is separated into two tubes (350 µl per tube) and 35 µl 3 M sodium acetate (pH:5.2) and 700 µl cold absolute ethanol are added to each tube. Tubes are mixed by inversion and are placed at 20°C for 30 minutes. After spinning for 15 minutes in a microfuge, the alcohol is removed and the pellet is washed with 1 ml 70 % ethanol at room temperature. Following a final spin for 5 minutes, the alcohol is removed with a micropipette and the tubes are left open on the bench for about 30 minutes for the evaporation of ethanol. Finally the DNA is resolubilized in 200 µl

of TE buffer (pH:8.0) by incubating at 56°C at least for 1 hour. This incubation can be done overnight when it is necessary to solubilize the pellet. The DNA samples are stored at -20°C.

2.2 DNA Isolation From Buccal Wash

Buccal wash sample is placed in 15 ml falcon tube and is centrifuged at 2500 rpm for 15 minutes. The supernatant is discarded and the pellet is dissolved in 500 µl WBCL Buffer by pipetting. The solution is then transferred to an eppendorf. After the addition of 13 µl 10 % SDS and 15 µl Proteinase K, the sample is incubated at 56°C overnight. The next day, 16.7 µl 5 M NaCl and 520 µl isoamylalcohol / chloroform (1 : 24) solution are added and centrifugation is applied at 13000 rpm for 10 minutes. Then, the supernatant is placed into a new tube and isoamylalcohol / chloroform (1 : 24) solution is added in 1:1 volume ratio. The sample is centrifuged at 13000 rpm for 10 minutes. This isoamylalcohol / chloroform extraction step is repeated once more and then, the supernatant is placed into another tube and isopropanol is added in twice the volume (1 :2). Following centrifugation at 13000 rpm for 10 minutes, the sample is washed with 500-1000 µl 70 % ethanol. After a final centrifugation for 10 minutes, the supernatant is discarded, 50 µl TE buffer is added and the tube is incubated at 56°C for 1-2 hours to solubilize the pellet. The DNA sample is stored at -20°C.

2.3 DNA Isolation From Skin Biopsy

In a sterile petri dish, the biopsy sample is rinsed three times with cold 1x PBS buffer. Blood and debri is removed with the help of a razor. It is then chopped into small pieces, taken into an eppendorf and 500 µl CVS buffer is added. The mixture is incubated at 56°C overnight. The following steps of the extraction procedure is the same as described above in section 2.1.

2.4 Polymerase Chain Reaction (PCR)

PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. There are 3 major steps in the reaction which are denaturation, annealing of primers and extension (synthesis). The requirements of the reaction are deoxynucleotides to provide both the energy and nucleosides for the synthesis of DNA, DNA polymerase, primer, template, and buffer containing magnesium. The deoxynucleotides and primers are present in excess, so the synthesis step can be repeated by heating the newly synthesized DNA to separate the strands and cooling to allow the primers to anneal to their complementary sequences. The heating and cooling cycles can be repeated and DNA will continue to accumulate exponentially until one of the reaction components are exhausted or the enzyme is unable to synthesize new DNA quickly enough. The number of cycles required for optimum amplification varies depending on the amount

of starting material and the efficiency of each amplification step (Mc Pherson *et al.*, 1991).

For the evaluation of chimerism status, 93 DNA samples which belong to 22 recipient-donor pairs were analyzed by PCR at 4 genomic STR loci (HUMVWA31, HUMTHO1, HUMF13A01, and HUMFES / FPS) and one PAH VNTR locus. Primer information is given above in Table 12. PCR reactions were performed in an automated thermal cycler (Perkin Elmer 9600).

Preparation of Reaction Mixture

The components of the reaction mixture and their amounts in 50 µl reaction volume are summarized in Table 13.

Table 13 : The Components of PCR Reaction Mixture

Reagent	**Final Concentration**	**Quantity for 50 µl mixture**
Sterile ddH_2O	-	variable
10x PCR Buffer	1x	5 µl
dNTP Mix (10 mM each)	0.2 mM	1 µl
Primer I	25 pmol	Variable (1 µl)*
Primer II	25 pmol	
Taq Polymerase (4u / µl)	1.2 u	0.30 µl
$MgCl_2$ (25 mM)	1.5 mM	3 µl
Template (DNA)	variable	2 µl

* The primer working solutions were prepared as 25 pmol / µl.

Amplification Conditions

The amplification conditions and the number of cycles for STR and PAH VNTR analyses are the following :

STR : 95°C 5 min / 94°C 1 min - 54°C 1 min - 72°C 1 min (10 cycles) / 90°C 1 min - 54°C 1 min - 72°C 1min (20 cycles) / 72°C 10 min.

PAH VNTR : 94°C 5 min / 94°C 30 sec. - 54°C 30 sec - 72°C 30 sec (30 cycles) / 72°C 5 min.

2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separation, identification, and purification of DNA fragments. The procedure can be divided into three stages :

1. A gel is prepared with an appropriate agarose concentration for the size of DNA fragments to be separated.
2. DNA samples are loaded into the sample wells and the gel is run at a voltage and for a time period to achieve optimal separation, and
3. The gel is stained, or if ethidium bromide is incorporated into the gel and electrophoresis buffer, it is visualized directly upon illumination with UV light.

Agarose concentration, applied voltage, electrophoresis buffers, and DNA conformation are among the parameters that affect the migration of DNA through agarose gels (Ausubel *et al.*, Vol 1: 2.5.1-2.5.9).

Isolated DNA samples and amplification products were run on agarose gels, 1.2 % and 2 (also 3 and 4) % gels were prepared respectively and 5 µl DNA or 5 µl amplification products were mixed with one volume loading buffer and were loaded onto gels. The gels were run in 1x TBE running buffer at voltages between 80V and 100V for different time intervals (30 min - 60 min). Standard DNA markers, 100 bp DNA ladder and ϕX175 HinfI were used to estimate the sizes of the fragments. The gels were stained further with ethidium bromide when necessary ; were visualized under UV light and photographed.

2.5.1 MetaPhor Agarose Gel Electrophoresis

The procedure is as described by the manufacturer. MetaPhor agarose is weighed according to the percentage and the amount of the gel. A beaker or flask that is 2.5 - 4 times the volume of the final agarose solution should be preferred. Chilled 1x TBE is added with a stir bar and the solution is slowly stirred to prevent the formation of lumps. The stir bar is removed, the flask is covered with plastic wrap, and a small hole is pierced as a steam vent. The solution is placed in a microwave oven which is adjusted to medium power. The solution is heated using several 20-60 sec intervals

and swirling is done between pulses. This heating-swirling cycles are done till agarose is completely dissolved. Then, plastic wrap is removed and warm deionised water is added to obtain initial volume of solution and is mixed. The solution is cooled to 60°C-70°C before pouring. The gel is left for polymerization at room temperature for 30 minutes and at 4°C for 30 minutes more. Finally the samples are loaded and the gel is run. It can be run at room temperature.

2 % metaphor agarose gel was prepared for the separation of STR alleles and the gel was run at 100V for 4 hours, stained with 1 μg/ml ethidium bromide solution for 40 minutes and destained with deionised water for 1 hour.

2.5.2 Formaldehyde - Agarose Gel Electrophoresis

The gel is prepared by melting the appropriate amount of agarose in water, cooling it to 60°C. 5x formaldehyde-gel running buffer and formaldehyde are added to give final concentrations of 1x and 2.2M, respectively. In order to achieve these concentrations, one part of a stock 12.3 M formaldehyde solution is diluted with 3.5 parts of agarose in water and 1.1 parts of 5x formaldehyde gel running buffer. The gel is casted in a chemical hood because formaldehyde vapors are toxic. The gel is allowed to set for at least 30 minutes at room temperature. Then the samples are prepared by mixing the following in a sterile microfuge tube:

RNA (up to 30 μg)	4.5 μl
5x formaldehyde gel-running buffer	2.0 μl
Formaldehyde	3.5 μl
Formamide	10.0 μl

The samples are incubated for 15 minutes at 65°C,and they are chilled on ice. Then, 2 μl of sterile DEPC-treated formaldehyde gel-loading buffer is added. Before loading the samples, the gel is prerun for 5 minutes at 5 V/cm. The gel is loaded and run in 1x formaldehyde gel-running buffer at 3-4 V / cm. When the bromophenol blue migrates approximately 8 cm the run is ended (Sambrook *et al.*, Vol 1: 7.43-7.45).

1.2 % gel was prepared, run at 50V for 5 hours, and then was stained with ethidium bromide (0.5 μg/ml) in 0.1M ammonium acetate for 30-45 minutes.

2.6 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE offers high resolution of low-molecular weight nucleic acids. A polyacrylamide gel is formed by the polymerization of acrylamide monomers into long chains, which are further covalently attached by a cross-linking agent N'N-methylene-bisacrylamide. Polymerization reaction is initiated by free radicals provided by APS and stabilized by TEMED. The pore size of a polyacrylamide gel is determined by the total percentage of acrylamide (Ausubel *et al.*, Vol 1: 2.7.1-2.7.5).

Amplified products are electrophoressed through 8-12 % denaturing polyacrylamide gel. The procedure is as follows:

50 ml of 12% denaturing gel solution is prepared and poured. The gel is allowed to polymerize for at least 1 hour and is then prerun at 90V for 30-60 minutes. The samples are mixed with 2x loading buffer in 1:1 volume ratio and are denatured at 95°C for 10 minutes. Then they are chilled on ice and are loaded onto the gel. The gel is run in 1x TBE running buffer at 90-100V for 18-20 hours and is silver stained.

2.7 Silver Staining

After the electrophoresis, the gel clamps are loosen and the glass plates are removed from the apparatus. The gel is placed in a plastic tray and is rinsed twice in ddH_2O for 1 minute. Then the gel is incubated in 300 ml of fixative solution for 3 minutes. The solution is discarded and the gel is treated with 300 ml of fresh fixative solution for the second time for 3 minutes. After rinsing with ddH_2O, the gel is incubated in 300 ml of 0.1 % $AgNO_3$ solution for 10 minutes at room temperature. Following this staining step, the gel is rinsed twice in ddH_2O rapidly. Finally 300 ml of ice-cold developer solution is added and the gel is incubated in this solution till the bands develop. Formaldehyde should be added just before the use of the solution. The development takes about 40 minutes. Then the developer solution is discarded and the gel is placed in 7.5 % acetic acid solution to stop the reaction. The gel is sandwiched between two transparency sheets for storage.

2.8 White Blood Cell Separation with Ficoll Centrifugation

In a 15 ml falcon tube, 2 ml bone marrow or peripheral blood sample is mixed with 2 ml 1x PBS buffer and is mixed gently by inversion. In another tube, 4 ml Ficoll separating solution is placed and the sample - PBS mixture is layered carefully on Ficoll. The sample is centrifuged at 2000 rpm for 30 minutes. The interphase is removed gently and is placed in a new tube. 5 ml 1x PBS is added, mixed and centrifuged at 1200 rpm for 7 minutes. This washing step can be repeated if necessary. After the centrifugation the pellet is kept in 1 ml Solution D and is stored at -80°C for further use.

2.9 RNA Isolation Using TRI Reagent

One of the most important aspects in the isolation of RNA is to prevent any degradation of RNA during the isolation procedure. Some agents that are used to preserve RNA integrity during extraction are summarized in Table 14.

Table 14: Agents Used to Preserve RNA Integrity During Extraction

Agent	Method of Ribonuclease Inactivation
DEPC	Alkylates proteins, disrupting protein structure
Guanidium thiocyanate	Denatures proteins
Phenol / chloroform	Extracts and denatures ribonucleases

(Adapted from Jones *et al.*, 1994)

TRI Reagent is a mixture of guanidine thiocyanate and phenol in a mono-phase solution which enables the simultaneous isolation of RNA, DNA, and proteins. RNA isolation procedure using TRI reagent is of 2 stages: sample preparation, and RNA isolation. The procedure is as described by the manufacturer.

Sample Preparation:

500 µl of sample (WBC preparation kept in Solution D, bone marrow or peripheral blood samples can be used directly as well) is mixed with 1 ml TRI reagent and a homogeneous lysate is formed by pipetting. It is centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant contains RNA and proteins. The clear supernatant is transferred to a new tube and is allowed to stand for 5 minutes at room temperature. Then, 200 µl of chloroform is added per ml of TRI reagent used, is shaked vigorously for 15 seconds and is allowed to stand at room temperature for 2-15 minutes. The centrifugation step at 12000 rpm for 15 minutes at 4°C separates the mixture in 3 phases:

i. red organic phase	protein
ii. interphase	DNA
iii. colorless upper aqueous phase	**RNA**

RNA Isolation :

The upper aqueous phase is transferred to a new tube,500 µl isopropanol is added per ml of TRI reagent used and is mixed. The sample is allowed to stand for 10 minutes at room temperature and then it is incubated at 4°C overnight. The next day, it is centrifuged at 12000 rpm for 15 minutes at 4°C. RNA precipitate forms a pellet on the side and bottom of the tube (interphase and organic phase can be stored at 4°C for subsequent DNA and protein isolation). The supernatant is removed and the RNA pellet is washed with 1 ml 75 % ethanol per ml of TRI reagent used. The sample is then vortexed and centrifuged at 7500 rpm for 5 minutes at 4°C. If RNA pellet floats, the wash is performed in 75 % ethanol at 12000 rpm for 10 minutes. The supernatant is discarded and the pellet is briefly dried for 5-10 minutes by air drying or under a vacuum. It is important not to overdry the pellet as this will greatly decrease its solubility. The usage of Speed-Vac is not recommended. Finally, an appropriate amount (50 µl) formamide, DEPC-water or 0.5 % SDS solution is added to the RNA pellet. To facilitate dissolution, repeated pipetting is applied at 55-60°C for 10-15 minutes. The isolated RNA sample can be kept at -20°C or -80°C.

For the isolation procedure, WBC preparation is used and is observed to give better results than bone marrow and peripheral blood. RNA pellet is dissolved in DEPC-ddH_2O and is kept at -80°C. After the isolation, the concentration of the samples were determined by spectrophotometric measurements. It has been stated that the 260 / 280 OD ratio of the RNA preparation should be no less than 1.7. The integrity of the isolated RNA samples is also checked by electrophoresis through 1.2 % gels containing formaldehyde.

2.10 First Strand cDNA Synthesis

Synthesis of first strand cDNA that is suitable for PCR amplification was performed with MBI Fermentas kit. The procedure is described by the manufacturer as follows:

For a total reaction volume of 20 µl:

The following reaction mix is prepared in a tube on ice:

- *Template RNA*

Total RNA	1-5 µg
or	
Poly A RNA	0.1-0.5 µg
or	
Specific RNA	0.01 pg-0.5 µg

- *Primer*

  Random hexamer (0.2 µg / µl) 1 µl

  or

  OligodT Primer (0.5 µg / µl) 1 µl

  Sequence specific 15-20 pmol

- *dd H_2O, nuclease free* up to 11 µl

This mixture (maximum volume should be 11 µl) is mixed gently and spinned down in a microfuge. It is then incubated at 70°C for 5 minutes, chilled on ice and the drops are collected by brief centrifugation. The tube is placed on ice and the following components are added in the indicated order:

- *5x reaction buffer* 4 µl
- *Ribonuclease inhibitor* (20 u / µl) 1 µl
- *10 mM dNTP mix* 2 µl

This mixture is mixed gently and spinned down in a microfuge. The next is the incubation at 37°C for 5 minutes. If random hexamer is used, the incubation is at 25°C for 5 minutes. Then,

- *M-MuLV RT* (20 u / µl) 2 µl

is added and the total volume becomes 20 µl. The mix is incubated at 37°C for 60 minutes. If random hexamer is used, it is incubated at 25°C for 10 minutes first and then at 37°C for 60 minutes. Finally, the reaction is stopped by heating at 70°C for 10 minutes. The sample is chilled on ice. The synthesized cDNA is stored at -20°C and it can directly be used for amplification by PCR.

In this study, total RNA that is isolated by TRI reagent was used as template and it was primed by random hexamer primers. The protocol was followed accordingly.

2.11 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Problems with PCR arise especially when dealing with poor quality or low-copy number nucleic acid template. Using nested PCR (nPCR) or nested RT-PCR (nRT-PCR), the sensitivity and specificity of both DNA and RNA amplification is considerably improved. The process utilizes two consecutive PCRs. The first PCR contains an external pair of primers, while the second contains two nested primers which are internal to the first primer pair, or one of the first primers and a single nested primer (half nested PCR). The larger fragment produced by the first reaction is used as template for the second PCR (Mc Pherson *et al.*, 1991).

For the detection of fusion transcripts, a panel was prepared regarding the type of the malignancy and the chromosomal rearrangements that are expected. The RT-PCR analyses were done according to this panel which is summarized in Table 15. A total of translocations and 1 inversion were studied which are:

1. t (9;22) (q34;q11)
2. t (8;21) (q22;q22)
3. t (15;17) (q22;q21)
4. t (4;11) (q21;q23)
5. t (1;19) (q23;p23)
6. inv 16 (p13;q22)

Table 15: Panel For RT-PCR Analysis

Disease	Number of Patients	Chromosomal Rearrangement Analyzed
CML	26	t (9;22)
AML	14	t (9;22), t (8;21), t (15;17), inv 16
MDS	3	t (9;22), t (8;21), t (15;17), t (4;11), t (1;19), inv 16
ALL	1	t (9;22), t (4;11), t (1;19)

Preparation of Reaction Mixture

The components of the reaction mixture and their amounts in 30 µl reaction volume are summarized in Table 16. Detailed primer information is given in Table 12.

Table 16: The Components of RT-PCR Reaction Mixture

Reagent	Final Concentration	Quantity for 30 µl mixture
Sterile ddH_2O	-	variable
10x PCR Buffer	1x	3 µl
DNTP Mix (10 mM each)	0.2 mM	0.6 µl
Primer I	20 pmol	Variable (1 µl)*
Primer II	20 pmol	
Taq Polymerase (4u / µl)	1.0 u	0.25 µl
$MgCl_2$ (25 mM)	1.5 mM	1.8 µl
Template (cDNA/1st PCR Product)	variable	4 µl

* The primer working solutions were prepared as 20 pmol / µl.

Amplification Conditions For Each Analysis

t (9;22) (q34;q11) :

1st PCR : 94°C 2 min / 93°C 3 min - 65°C 2 min - 72°C 3 min (30 cycles) / 65°C 2 min - 72°C 15min (1 cycle)

2nd PCR : 94°C 2 min / 93°C 2 min - 65°C 1 min - 72°C 2 min (30 cycles) / 65°C 1 min - 72°C 15min (1 cycle)

t (8;21) (q22;q22) :

1st PCR and 2nd PCR : 94°C 2 min - 55°C 1min - 72°C 1 min (1 cycle) / 94°C 1 min - 55°C 1min - 72°C 1 min (14 cycles) / 94°C 1 min - 55°C 1min - 72°C 2 min (24 cycles) / 94°C 1 min - 55°C 1min - 72°C 7 min (1 cycle)

t (15;17) (q22;q21) :

1st PCR and 2nd PCR : 95°C 5 min / 95°C 1 min - 55°C 1min - 72°C 1 min (30 cycles)

t (4;11) (q21;q23) :

1st PCR and 2nd PCR : 94°C 2 min / 94°C 1 min - 56°C 1min - 72°C 1 min (30 cycles)

t (1;19) (q23;p23) : (Single PCR)

94°C 2 min / 94°C 30 sec - 62°C 30 sec - 72°C 30 sec (40 cycles)

inv 16 (p13;q22) :

1st PCR and 2nd PCR : 95°C 5 min / 94°C 1 min - 58°C 1min - 72°C 1 min (45 cycles)

Beta-actin gene was amplified to check the integrity of the cDNA samples. The amplification was both performed at separate tubes and in the same tube with each of the above analyses. The amplification condition as a separate reaction is as follows:

Beta-Actin (TC66 - TC67) :

95°C 3 min / 94°C 1 min - 60°C 1min - 72°C 1 min (30 cycles)

III. RESULTS

1. Evaluation of Chimerism with DNA Polymorphisms After Allogeneic Transplantation

In this study, polymorphic DNA markers, four STR and one PAH VNTR polymorphisms, were used to evaluate the chimeric status of 22 donor-recipient pairs after allogeneic transplantation. For this purpose, genomic DNA was isolated from buccal wash, and peripheral blood samples (also skin biopsy sample of one patient, KIT-4) of recipients and peripheral blood samples of donors. Then, PCR amplification was performed with specific STR and PAH primers. The amplified products were run on denaturing polyacrylamide gels and silver stained for visualization. Genotype analysis was done at intervals ranging from 15 to 2920 days post-transplantation. The original genotype (pre-transplantation) of the recipient was determined from buccal wash samples. The post-transplantation genotype of the recipient and the original genotype of the donor were determined from peripheral blood samples. The chimerism status is determined by comparison of the recipient's post-transplantation genotype with the pre-transplantation genotype and the genotype of the donor.

Complete chimerism (CC) was documented in 12 patients (KIT-5, KIT-6, KIT-17, KIT-18, KIT-19, KIT-21, KIT-22, KIT-23, KIT-24, KIT-25, KIT-29, and KIT-32), and mixed chimerism (MC) in one patient (KIT-4). Rejection was shown in two β-TM patients (KIT-15, KIT-16), and in one CML patient (KIT-30) (Table 18). Chimerism was evaluated initially using the STR polymorphisms. PAH polymorphism was used for uninformative cases with STR polymorphism typing. In 22 recipient-donor pairs, THO1 was informative in ten pairs (KIT-5, KIT-6, KIT-15, KIT-16, KIT-18, KIT-21, KIT-22, KIT-24, KIT-25, and KIT-32); F13A01 in five pairs, (KIT-15, KIT-16, KIT-21, KIT-23, and KIT-29); vWA in four pairs, (KIT-4, KIT-16, KIT-17, and KIT-21); and FES /FPS in three pairs (KIT-6, KIT-19, and KIT-21). PAH was analyzed in ten cases, and was informative in two of them (KIT-23, KIT-30) (Table 17). The chimerism status of 11 pairs (KIT-4, KIT-5, KIT-17, KIT-18, KIT-19, KIT-22, KIT-24, KIT-25, KIT-29, KIT-30, and KIT-32) were determined based on the information at one locus; three pairs (KIT-6, KIT-15, and KIT-23) at two loci; one pair (KIT-16) at three loci; and one pair (KIT-21) at four loci. For the remaining six recipient-donor pairs (KIT-1, KIT-10, KIT-11, KIT-12, KIT-13, and KIT-14) all five loci were non-informative (Table 18).

Table 17 : Analyzed Polymorphic Loci for Chimerism Evaluation

Case	PAH	THO1	FES / FPS	vWA	F13A01	Results (+ days)
KIT-1	-	-	-	-	-	NI (+180)
KIT-4	-	-	-	+	-	MC (+491)
KIT-5		+	-	-	-	CC (+153)
KIT-6		+	+	-	-	CC (+590)
KIT-10	-	-	-	-	-	NI (+132)
KIT-11	-	-	-	-	-	NI (+187)
KIT-12	-	-	-	-	-	NI (+1042)
KIT-13	-	-	-	-	-	NI (+?)
KIT-14	-	-	-	-	-	NI (+367)
KIT-15		+	-	-	+	RJC (+270)
KIT-16		+	-	+	+	RJC (+605)
KIT-17	-	-	-	+	-	CC (+75)
KIT-18		+	-	-	-	CC (+64)
KIT-19		-	+	-	-	CC (+39)
KIT-21		+	+	+	+	CC (+2920)
KIT-22		+	-	-	-	CC (+469)
KIT-23	+	-	-	-	+	CC (+15)
KIT-24		+	-	-	-	CC (+45)
KIT-25		+	-	-	-	CC (+15)
KIT-29		-	-	-	+	CC (+118)
KIT-30	+	-	-	-	-	RJC (+140)
KIT-32		+	-	-	-	CC (+44)

NI : Non-Informative

RJC : Rejection

+: Informative

-: Not informative

Table 18 : STR and PAH VNTR Analyses for Chimerism Evaluation

Case	Disease	Time of PCR Analysis (days)	Informative Loci	Chimeric Status
1. KIT-1	CML (Ph+)	+54,+180	none	Unknown
2. KIT-4	AML	+456, +491, +140, +230 (2 BMT)	VWA	MC
3. KIT-5	AML (M6)	+50, +153	THO1	CC
4. KIT-6	AML	+590	THO1, FES/FPS	CC
5. KIT-10	AML	+15, +132	none	Unknown
6. KIT-11	AML (M6)	+187	none	Unknown
7. KIT-12	AML	+1042	none	Unknown
8. KIT-13	CML	+?	none	Unknown
9. KIT-14	AML (M2)	+367	none	Unknown
10. KIT-15	β-TM	+60, +180, +270	F13, THO1	Rejection
11. KIT-16	β-TM	+395, +485, +605	VWA, THO1, F13	Rejection
12. KIT-17	NHL	+14, +60, +75	VWA	CC
13. KIT-18	CML	+38, +64	THO1	CC
14. KIT-19	AML	+39	FES/FPS	CC
15. KIT-21	AML	+2920	VWA,THO1,FES,F13	CC
16. KIT-22	AML(M3)	+469	THO1	CC
17. KIT-23	MDS + AML	+15	PAH, F13	CC
18. KIT-24	AML	+45	THO1	CC
19. KIT-25	AML	+15	THO1	CC
20. KIT-29	CML	+118	F13	CC
21. KIT-30	CML	+1, +23, +51, +140	PAH	Rejection
22. KIT-32	CML	+2, +44	THO1	CC

CML: Chronic myelogenous leukemia
AML: Acute myelogenous leukemia
MDS: Myelodysplastic syndromes
NHL: Non Hodgkin's lymphoma
β-TM: β-thalassemia
MC : Mixed chimeric
CC: Complete Chimeric
+ : Number of days after-transplantation

1.1 Visualization of DNA Samples

Isolated DNA samples were run on 1.2 % agarose gels.

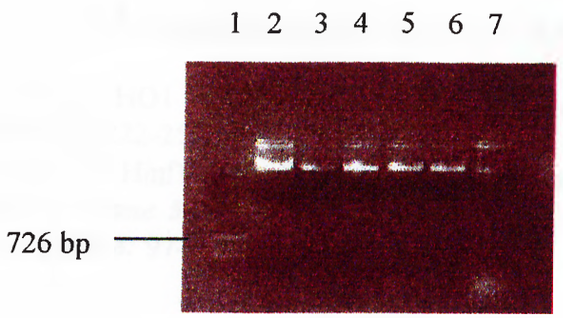

Figure 11 : Patient DNA Samples

Lane 1: ϕX 174 HinfI, ***Lane 2:*** 97-325 (KIT 19), ***Lane 3:*** 97-326 (KIT 19)
Lane 4: 97-348 (KIT 22), ***Lane 5:*** 97-381 (KIT 23), ***Lane 6:*** 97-555 (KIT 18)
Lane 7: 97-558 (KIT-37)

1.2 Polymerase Chain Reaction

Isolated DNA samples were amplified by PCR with STR and PAH loci specific primers. STR amplification products were run on 2 % agarose gel before PAGE (Figure 12). PAH VNTR amplification products were run on 3 % agarose gel instead of polyacrylamide gels as the polymorphism was 30 nucleotide repeats. (Figure 13). MetaPhor agarose gel electorphoresis enabled the separation of STR alleles without application of PAGE (Figure 14).

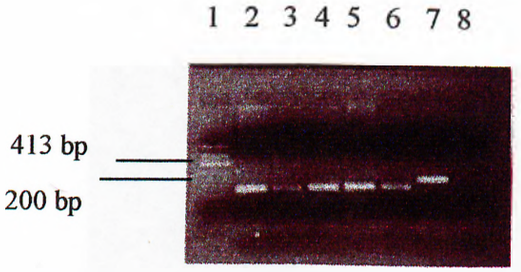

Figure 12 : PCR Amplification of THO1 and FES / FPS Loci

Lanes 2-6 are THO1 amplifications (179-203 bp), and Lanes 7 and 8 are FES / FPS amplifications (222-250 bp).
Lane 1: ϕX 174 HinfI, ***Lane 2:*** 97-35 (KIT 5), ***Lane 3:*** 97-315 (KIT 5), ***Lane 4:*** 97-500 (KIT 5), ***Lane 5:*** 97-36 (KIT 5), ***Lane 6:*** 97-563 (KIT 37), ***Lane 7:*** 97-147 (KIT 10), ***Lane 8:*** 97-148 (KIT 10)

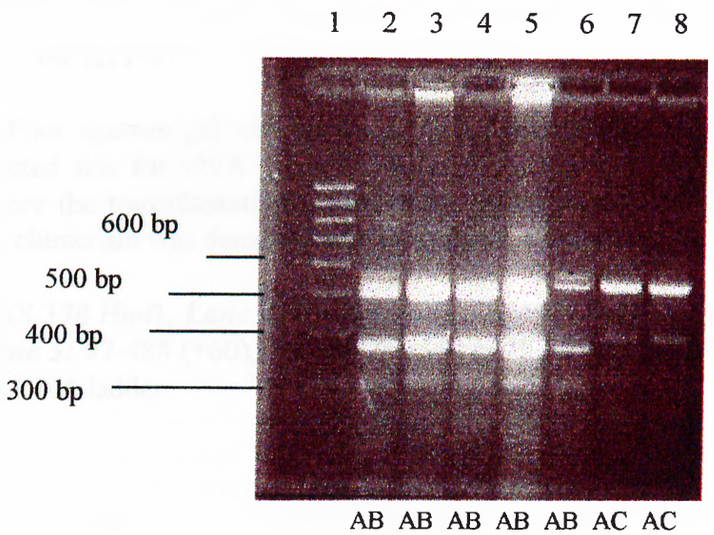

Figure 13: PCR Amplification of PAH VNTR Locus for KIT-2 and KIT-23

Expected sizes could be 380 bp, 470 bp, 500 bp, 530 bp, 560 bp, or 650 bp according to the number of copies of the 30 nucleotide repeats. Lanes 2-5 represent samples of KIT-2, and Lanes 6-8 represent samples of KIT-23. + indicates number of days in post transplantation period. Possible genotypes are indicated below the figure. While KIT-2 is non-informative, KIT-23 is complete chimeric for PAH VNTR locus.

Lane 1: 100 bp DNA ladder, ***Lane 2:*** 97-565 (pre), ***Lane 3:*** 97-18 (+62), ***Lane 4:*** 97-566 (+203), ***Lane 5:*** 97-20 (donor), ***Lane 6:*** 97-381 (pre), ***Lane 7:*** 97-494 (+15), ***Lane 8:*** 97-403 (donor)

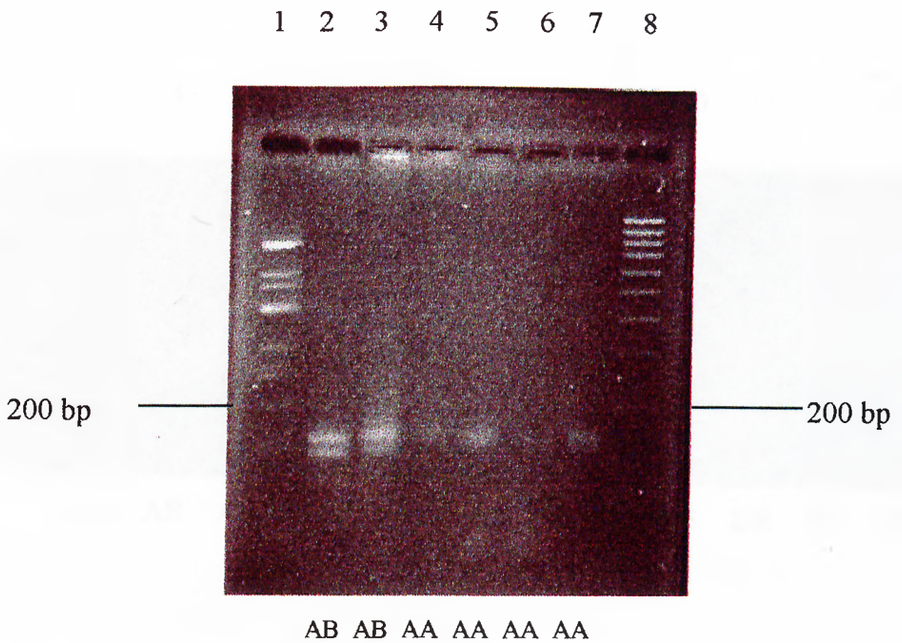

Figure 14: MetaPhor Agarose Gel Showing Complete Chimerism at vWA Locus for KIT-17

2% MetaPhor agarose gel was prepared for visualization of amplification products. The expected size for vWA locus is 139-167 bp. Pre indicates the patient sample taken before the transplantation. Possible genotypes are indicated below the figure. Complete chimerism was demonstrated for KIT-17 at vWA locus.

Lane 1: ϕX 174 HinfI, ***Lane 2:*** 97-320 (pre), ***Lane 3:*** 97-321 (pre), ***Lane 4:*** 97-380 (+14), ***Lane 5:*** 97-488 (+60), ***Lane 6:*** 97-548 (+75), ***Lane 7:*** 97-549 (donor), ***Lane 8:*** 100 bp DNA ladder

1.3 Polyacrylamide Gel Electrophoresis

After PCR products are checked on agarose test gels, they are run on 8-12 % denaturing polyacrylamide gels and the analysis is completed after silver staining. In the following figures, different outcomes are shown : Rejection (Figure 15), complete chimeric (CC) (Figure 16, Figure 17), and non-informative (NI) (Figure 18).

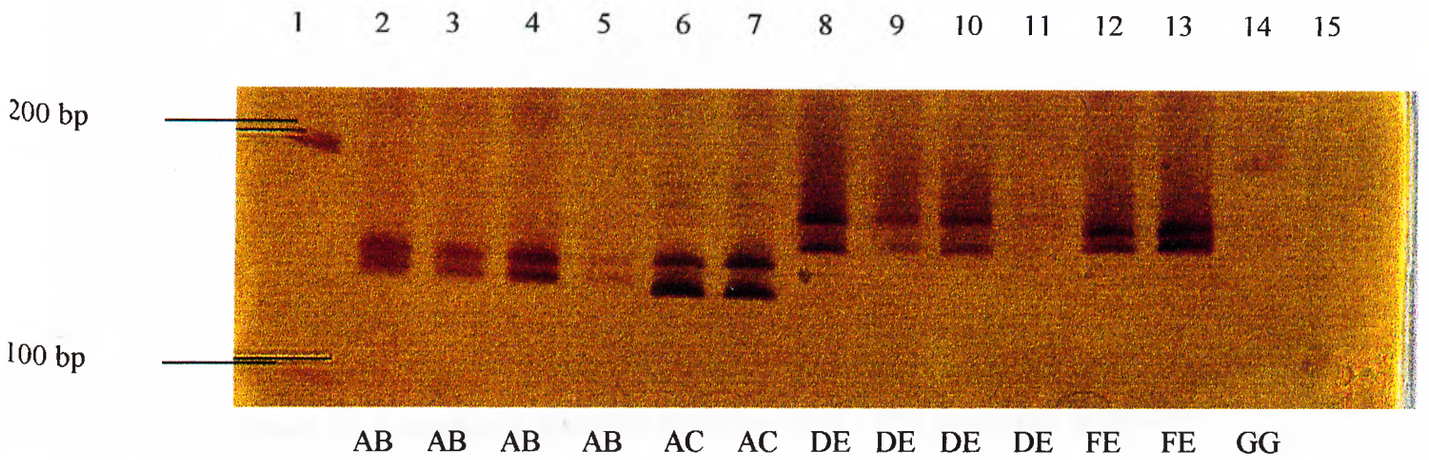

Figure 15: Rejection Based on vWA and THO1 Genotype in KIT-16

8 % polyacrylamide gel was prepared for visualization of the amplified products. Lanes 2-7 indicate amplified samples for vWA locus and Lanes 8-13 indicate samples amplified for THO1 locus. Lane 14 indicates a positive control sample for F13A01 locus with known homozygous genotype, and Lane 15 indicates negative control. The expected sizes for vWA, THO1, and F13A01 are 139-167 bp, 179-203 bp, and 283-331 bp respectively. Possible genotypes are indicated below the figure. Rejection was demonstrated for KIT-16 at vWA and THO1 loci.

Lane 1: 100 bp DNA ladder,
Lane 2: 97-384 (pre), ***Lane 3:*** 97-316 (+395), ***Lane 4:*** 97-317 (+485), ***Lane 5:*** 97-385 (+605), ***Lane 6:*** 97-318 (donor), ***Lane 7:*** 97-319 (donor), (vWA)
Lane 8: 97-384 (pre), ***Lane 9:*** 97-316 (+395), ***Lane 10:*** 97-317 (+485), ***Lane 11:*** 97-385 (+605), ***Lane 12:*** 97-318 (donor), ***Lane 13:*** 97-319 (donor), (THO1)
Lane 14: positive control sample (F13A01), ***Lane 15:*** negative control

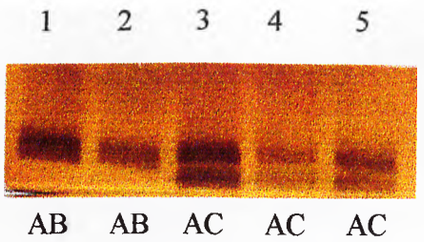

Figure 16: Complete Chimerism Based on FES / FPS Genotype in KIT-19

12 % polyacrylamide was prepared for visualization of amplified fragments. Possible genotypes are indicated below the figure. Complete chimerism was demonstrated for KIT-19 at FES / FPS locus.

Lane 1: 97-324 (pre), ***Lane 2:*** 97-325 (pre), ***Lane 3:*** 97-509 (+39), ***Lane 4:*** 97-326 (donor), ***Lane 5:*** 97-510 (donor)

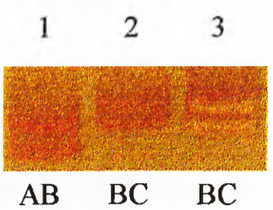

Figure 17: Complete Chimerism Based on THO1 Genotype in KIT-25

12 % polyacrylamide was prepared for visualization of amplified fragments. Possible genotypes are indicated below the figure. Complete chimerism was demonstrated for KIT-25 at THO1 locus.

Lane 1: 97-407 (pre), ***Lane 2:*** 97-495 (+15), ***Lane 3:*** 97-509 (donor)

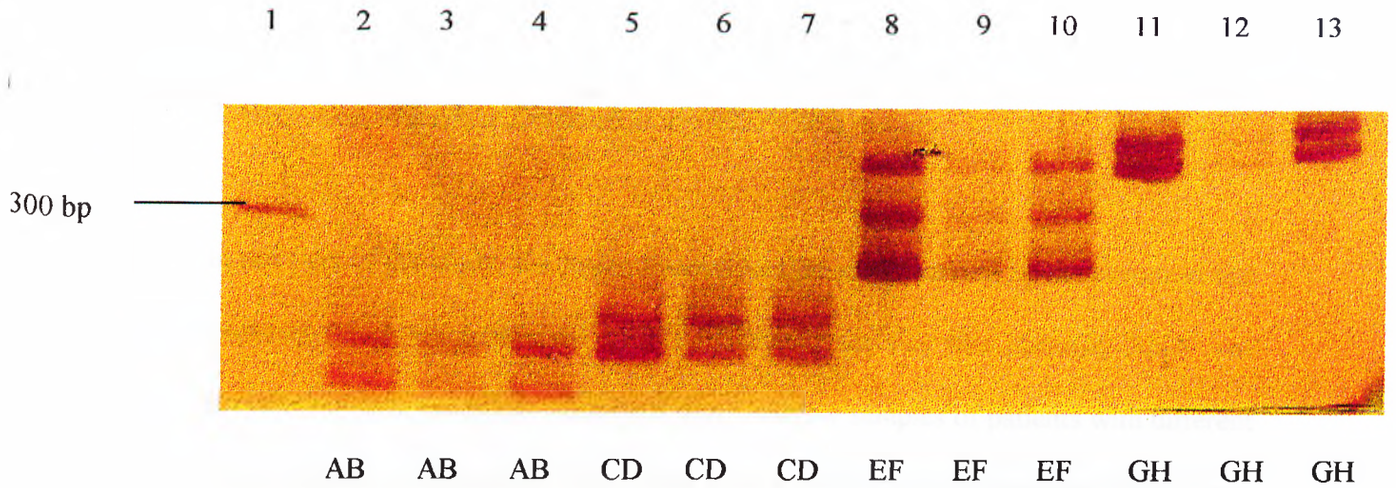

Figure 18: : Non-Informative Results at vWA, THO1, F13A01, and FES / FPS Loci in KIT-21

12 % polyacrylamide was prepared for visualization of amplified fragments. Possible genotypes are indicated below the figure. Lanes 2-4 indicate samples for vWA amplified products, Lanes 5-7 for THO1 amplified products, Lanes 8-10 for F13A01 amplified products, and Lanes 11-13 for FES / FPS amplified products. KIT-21 was shown to be non-informative at all four loci analyzed. The expected sizes for vWA, THO1, F13A01, and FES / FPS are 139-167 bp, 179-203 bp, 283-331 bp, and 222-250 bp respectively.

Lane 1: 100 bp DNA ladder,
Lane 2: 97-337 (pre), ***Lane 3:*** 97-335 (+2920), ***Lane 4:*** 97-336 (donor), (vWA)
Lane 5: 97-337 (pre), ***Lane 6:*** 97-335 (+2920), ***Lane 7:*** 97-336 (donor), (THO1)
Lane 8: 97-337 (pre), ***Lane 9:*** 97-335 (+2920), ***Lane 10:*** 97-336 (donor), (F13A01)
Lane 11: 97-337 (pre), ***Lane 12:*** 97-335 (+2920), ***Lane 13:*** 97-336 (donor) (FES / FPS)

2. Detection of Fusion Transcripts by RT-PCR Analysis

In this part of the study, the fusion transcripts that are generated by the chromosomal rearrangements were detected by RT-PCR analysis. For this purpose, RNA was isolated from the peripheral blood and bone marrow samples of patients with different hematological malignancies. Then first strand cDNA was synthesized which was further amplified with translocation and inversion specific primers. The analysis was carried out according to the panel prepared (Table 15). There are five translocations and one inversion type rearrangement in the panel. A total of 49 samples which belong to 44 patients were analyzed. Nine of these patients underwent allogeneic transplantation. RT-PCR positivity for these patients indicates minimal residual disease. 17 patients are newly diagnosed for which RT-PCR positivity indicates the presence of the fusion transcripts that are characteristic for the malignancy involved.

Among 14 AML patients only one (FS-23) was shown to be positive for t (15;17). Four patients (FS-2, FS-8, FS-21, and FS-29) are positive for inv 16. Altered size fragments were observed in two patients (FS-8, FS-29) as 2^{nd} PCR products. These may represent different breakpoints in chromosome 16. Our single ALL patient (FS-3) is negative for all the translocations analyzed (9;22, 4;11, and 1;19). Among three MDS patients only two of them (FS-5, and FS-6) are positive for t (9;22) while the third one (FS-12) is negative for all of the six rearrangements analyzed (Table 19). It is interesting to note that among the nine translocation positive acute leukemia patients, seven of them (FS-8, FS-11, FS-14, FS-23, FS-26, FS-29, and FS-46) are

newly diagnosed (see also Table 10). This point stresses the importance of performing a fusion transcript detection test for the diagnosis of hematological malignancies.

Translocation 9;22 analysis was performed in a total of 26 CML patients. 14 of them are translocation positive while in 12 of them no fusion transcript was detected. Among the translocation positive patients, seven of them (FS-13, FS-17, FS-20, FS-30, FS-31, FS-35 and FS-43) are positive only for the 198 bp transcript, while three patients (FS-10, FS-24, and FS-42) are positive only for the 273 bp transcript. Four patients (FS-4, FS-16, FS-33, and FS-45) are shown to be positive for both of the t (9;22) transcripts (Table 20). Among the 14 translocation positive CML patients, eight of them (FS-10, FS-16, FS-27, FS-31, FS-33, FS-35, FS-38, and FS-45) are newly diagnosed (see also Table 10). All three patients that are under interferon treatment (FS-17, FS-20, FS-43) are positive for the 198 bp transcript. All seven patients (FS-22, FS-25, FS-28, FS-32, FS-34, FS-39, and FS-44) who underwent allogeneic PBSCT are negative for t (9;22) transcripts which is an indication of MRD negativity (Table 20).

Table 19 : RT-PCR Analysis for Detection of Fusion Transcripts in Acute Leukemia Patients

Patient Code	Clinical Diagnosis	t (9;22) *	t (15;17)	t (8;21)	inv 16	t (4;11)	t (1;19)	Result
1. FS-18	AML	-	-	-	-	NA	NA	TND
2. FS-47	AML	-	-	-	-	NA	NA	TND
3. FS-8	AML-M1	-	-	-	+	NA	NA	inv 16 +
4. FS-29	AML-M1	-	-	-	+	NA	NA	inv 16 +
5. FS-46	AML-M1	-	-	-	-	NA	NA	TND
6. FS-7	AML-M2	-	-	-	-	NA	NA	TND
7. FS-15	AML-M2	-	-	-	-	NA	NA	TND
8. FS-1	AML-Eo M3	NA	NA	NA	-	NA	NA	TND
9. FS-26	AML-M3	-	-	-	-	NA	NA	TND
10.FS-2	AML-M4	-	-	-	+	NA	NA	inv 16 +
11.FS-11	AML-M4	-	-	-	-	NA	NA	TND
12.FS-21	AML-Eo M4	-	-	-	+	NA	NA	inv 16 +
13.FS-23	AML-M4	-	+	-	-	NA	NA	t(15;17) +
14.FS-14	AML biphenotypic	-	-	-	-	NA	NA	TND
15.FS-3	ALL	-	NA	NA	NA	-	-	TND
16.FS-5	MDS-RAEBt	+ / +	-	-	-	-	-	t (9;22) +
17.FS-6	MDS-RAEB	+ / -	-	-	-	-	-	t (9;22) +
18.FS-12	MDS	-	-	-	-	-	-	TND

TND: Translocation not detected

NA: Analysis not applicable (Table 15)

* First + denotes for the positivity for 198 bp product and second + denotes positivity for 273 bp product in t (9;22).

Table 20 : RT-PCR Analysis for Detection of Fusion Transcripts in Chronic Myelogenous Leukemia (CML) Patients

Patient Code	t (9;22)	Result
1. FS-4	+ /+	198 bp +, 273 bp +
2. FS-9	- / -	translocation negative
3. FS-10	- / +	273 bp +
4. FS-13	+ / -	198 bp +
5. FS-16	+ /+	198 bp +, 273 bp +
6. FS-17	+ / -	198 bp +
7. FS-20	+ / -	198 bp +
8. FS-22	- / -	translocation negative
9. FS-24	- / +	273 bp +
10.FS-25	- / -	translocation negative
11.FS-27	- / -	translocation negative
12.FS-28	- / -	translocation negative
13.FS-30	+ / -	198 bp +
14.FS-31	+ / -	198 bp +
15.FS-32	- / -	translocation negative
16.FS-33	+ /+	198 bp +, 273 bp +
17.FS-34	- / -	translocation negative
18.FS-35	+ / -	198 bp +
19.FS-36	- / -	translocation negative
20.FS-38	- / -	translocation negative
21.FS-39	- / -	translocation negative
22.FS-40	- / -	translocation negative
23.FS-42	- / +	273 bp +
24.FS-43	+ / -	198 bp +
25.FS-44	- / -	translocation negative
26.FS-45	+ /+	198 bp +, 273 bp +

2.1 Visualization of RNA Samples

The isolated RNA samples were run on 1.2 % formaldehyde-containing agarose gels which were stained with ethidium bromide and destained in ddH_2O before they were photographed.

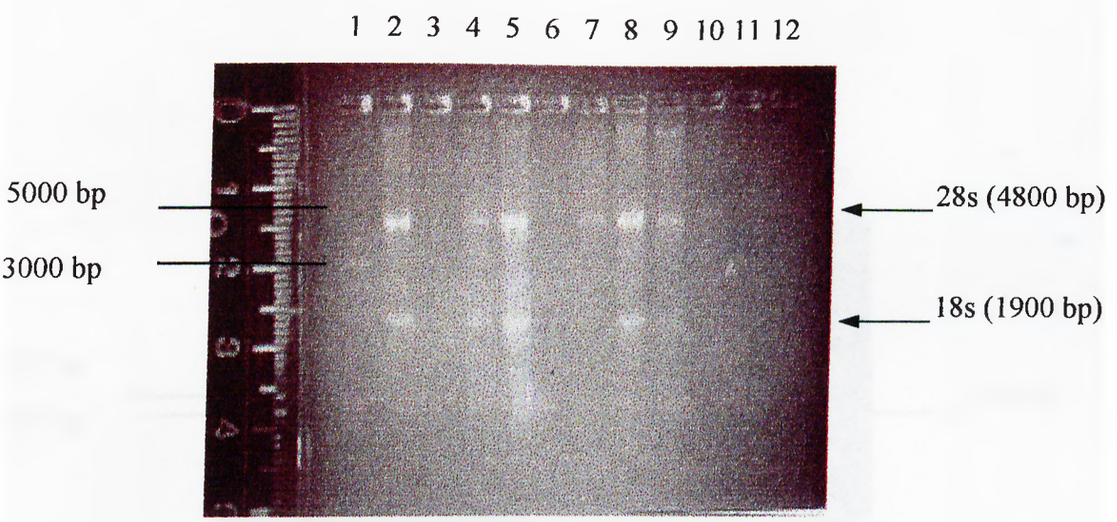

Figure 19: Patient RNA Samples

Lane 1: 1 kb DNA ladder, ***Lane 2:*** 98-53 (FS-13), ***Lane 3:*** 98-54 (FS-14), ***Lane 4:*** 98-55 (FS-15), ***Lane 5:*** 98-56 (FS-15), ***Lane 6:*** 98-57 (FS-16), ***Lane 7:*** 98-58 (FS-17), ***Lane 8:*** 98-61 (FS-18), ***Lane 9:*** 98-62 (FS-19), ***Lane 10:*** 98-63 (FS-20), ***Lane 11:*** 1 kb DNA ladder

2.2 cDNA Synthesis

The integrity of the synthesized cDNA samples was checked by the amplification of the *β-actin* locus by RT-PCR. The amplified fragment is 551 bp using primer pair TC-66 and TC-67 (Table 12). As depicted in Figures 20 A and 20 B cDNA synthesis of all the samples were successfully achieved and amplification was observed.

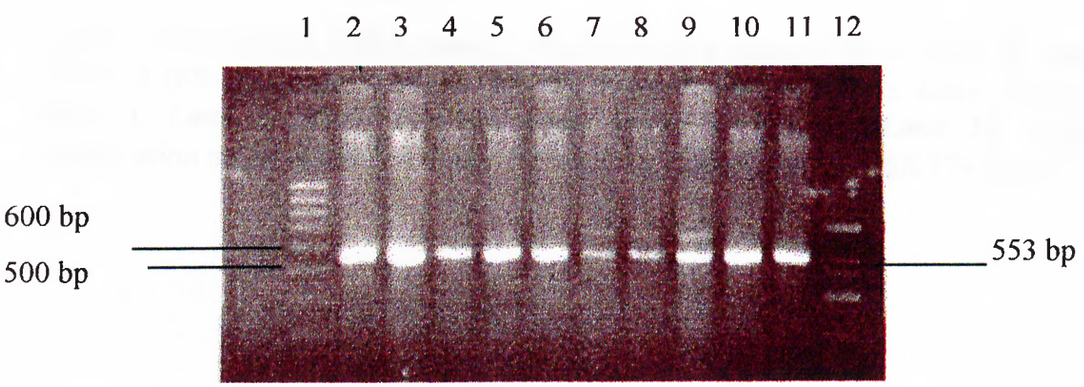

Figure 20 A : *β-Actin* Amplification of cDNA Samples

Lane 1: 100 bp DNA ladder, ***Lane 2:*** 97-664 (FS-1), ***Lane 3:*** 98-28 (FS-2), ***Lane 4:*** 98-29 (FS-3), ***Lane 5:*** 98-30 (FS-4), ***Lane 6:*** 98-31 (FS-4), ***Lane 7:*** 98-35 (FS-6), ***Lane 8:*** 98-36 (FS-7), ***Lane 9:*** 98-37 (FS-8), ***Lane 10:*** 98-45 (FS-9), ***Lane 11:*** 98-47 (FS-10), ***Lane 12:*** ϕX 174 HinfI

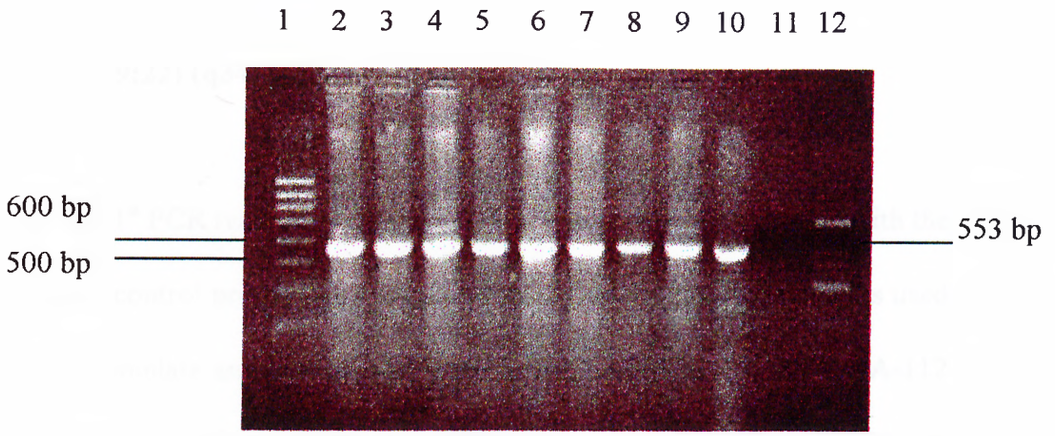

Figure 20 B : *β-Actin* Amplification of cDNA Samples

Lane 1: 100 bp DNA ladder, ***Lane 2:*** 98-48 (FS-11), ***Lane 3:*** 98-52 (FS-12), ***Lane 4:*** 98-53 (FS-13), ***Lane 5:*** 98-54 (FS-14), ***Lane 6:*** 98-55 (FS-15), ***Lane 7:*** 98-56 (FS-15), ***Lane 8:*** 98-57 (FS-16), ***Lane 9:*** 98-63 (FS-20), ***Lane 10:*** HT29 translocation negative cell line, ***Lane 11:*** negative control ***Lane 12:*** ϕX 174 HinfI

2.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For each of the chromosomal rearrangements analyzed, primer pairs, expected sizes are depicted in the schematic presentations and the corresponding results obtained are given in the following sections:

2.3.1 t (9;22) (q34;q11)

For the 1st PCR reaction, GA-114 / GA 111 pair was used together with the *β-actin* control primers. In the second step, this reaction mixture was used as the template and primed with inner primers which is GA-113 / GA-112 pair. Location of the oligomers is indicated in Figure 21 below. Due to the alternative splicing of *BCR* gene, two different fusion transcripts are generated as 198 bp and 273 bp respectively. As an additional internal control, GA-431 / GA-111 pair was also used and 202 bp fragment was successfully amplified (data not shown).

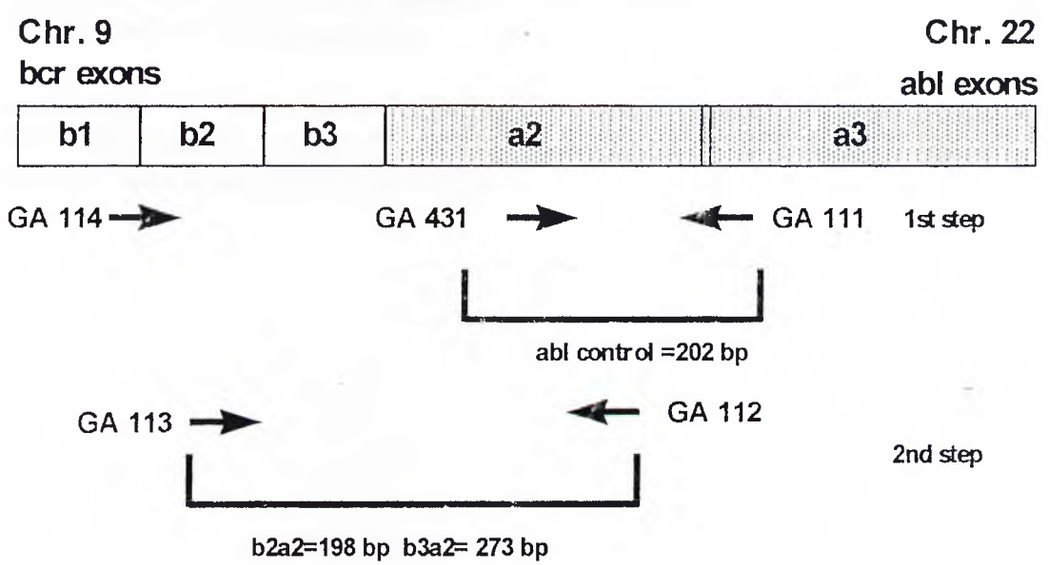

Figure 21: Schematic Representation of the Amplified Region for t (9;22)

(Adapted from Frenoy *et al.*, 1994)

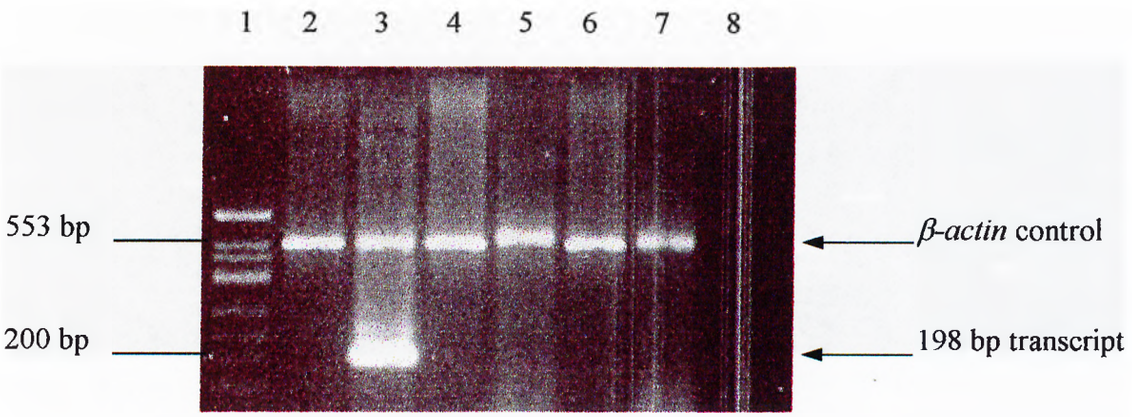

Figure 22 A: RT-PCR Amplification for t (9;22) q34;q11)

Lanes 2, 4, and 6 indicate 1st PCR reactions while Lanes 3, 5, and 7 indicate 2nd PCR reactions of the corresponding samples. Lane 8 is the negative control of the 2nd PCR reaction. Sample 98-58 (FS-17) was observed to be positive for the 198 bp b2a2 fusion transcript while other samples, 98-61 (FS-18) and 98-64 (FS-21), were negative for both of the transcripts.

Lane 1: ϕX 174 HinfI, ***Lane 2:*** 98-58 (1st), ***Lane 3:*** 98-58 (2nd) (FS-17), ***Lane 4:*** 98-61 (1st), ***Lane 5:*** 98-61 (2nd) (FS-18), ***Lane 6:*** 98-64 (1st), ***Lane 7:*** 98-64 (2nd) (FS-21), ***Lane 8:*** negative control (2nd)

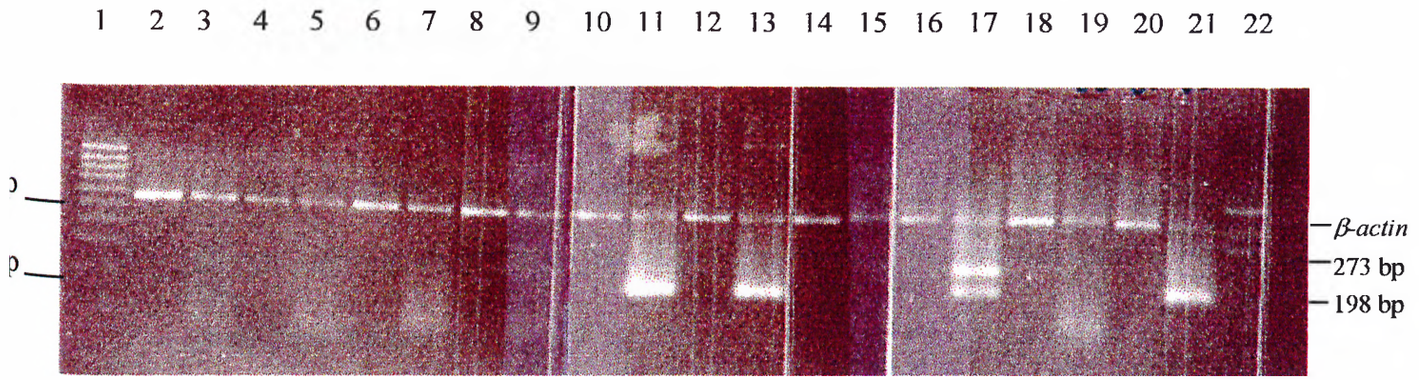

Figure 22 B: RT-PCR Amplification for t (9;22) (q34;q11)

Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 indicate 1st PCR reactions while Lanes 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 indicate 2nd PCR reactions of the corresponding samples. Samples 98-85 (FS-30), 98-86 (FS-31), and 98-90 (FS-35) were observed to be positive for the 198 bp b2a2 fusion transcript while other samples, 98-72 (FS-26), 98-79 (FS-27), 98-80 (FS-28), 98-84 (FS-29), 98-87 (FS-32), and 98-89 (FS-34) were negative for both of the transcripts. Sample 98-88 (FS-33) was observed to be positive for both b2a2 (198 bp), and the b3a2 (273 bp) fusion transcripts.

Lane 1: 100 bp DNA ladder,
Lane 2: 98-72 (1st), ***Lane 3:*** 98-72 (2nd) (FS-26),
Lane 4: 98-79 (1st), ***Lane 5:*** 98-79 (2nd) (FS-27),
Lane 6: 98-80 (1st), ***Lane 7:*** 98-80 (2nd) (FS-28),
Lane 8: 98-84 (1st), ***Lane 9:*** 98-84 (2nd) (FS-29),
Lane 10: 98-85 (1st), ***Lane 11:*** 98-85 (2nd) (FS-30),
Lane 12: 98-86 (1st), ***Lane 13:*** 98-86 (2nd) (FS-31),
Lane 14: 98-87 (1st), ***Lane 15:*** 98-87 (2nd) (FS-32),
Lane 16: 98-88 (1st), ***Lane 17:*** 98-88 (2nd) (FS-33),
Lane 18: 98-89 (1st), ***Lane 19:*** 98-89 (2nd) (FS-34),
Lane 20: 98-90 (1st), ***Lane 21:*** 98-90 (2nd) (FS-35),
Lane 22: ϕX 174 HinfI

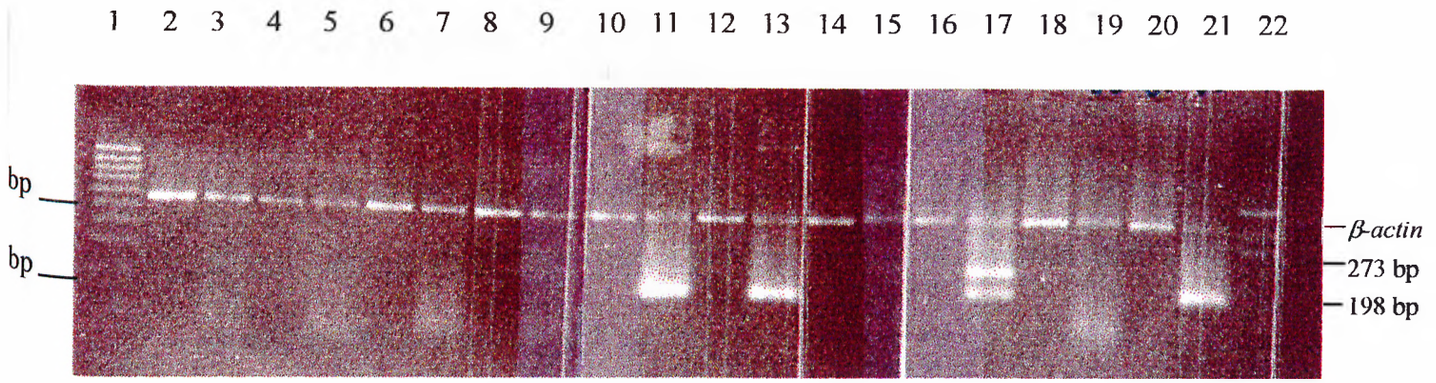

Figure 22 B: RT-PCR Amplification for t (9;22) (q34;q11)

Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 indicate 1st PCR reactions while Lanes 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 indicate 2nd PCR reactions of the corresponding samples. Samples 98-85 (FS-30), 98-86 (FS-31), and 98-90 (FS-35) were observed to be positive for the 198 bp b2a2 fusion transcript while other samples, 98-72 (FS-26), 98-79 (FS-27), 98-80 (FS-28), 98-84 (FS-29), 98-87 (FS-32), and 98-89 (FS-34) were negative for both of the transcripts. Sample 98-88 (FS-33) was observed to be positive for both b2a2 (198 bp), and the b3a2 (273 bp) fusion transcripts.

Lane 1: 100 bp DNA ladder,
Lane 2: 98-72 (1st), ***Lane 3:*** 98-72 (2nd) (FS-26),
Lane 4: 98-79 (1st), ***Lane 5:*** 98-79 (2nd) (FS-27),
Lane 6: 98-80 (1st), ***Lane 7:*** 98-80 (2nd) (FS-28),
Lane 8: 98-84 (1st), ***Lane 9:*** 98-84 (2nd) (FS-29),
Lane 10: 98-85 (1st), ***Lane 11:*** 98-85 (2nd) (FS-30),
Lane 12: 98-86 (1st), ***Lane 13:*** 98-86 (2nd) (FS-31),
Lane 14: 98-87 (1st), ***Lane 15:*** 98-87 (2nd) (FS-32),
Lane 16: 98-88 (1st), ***Lane 17:*** 98-88 (2nd) (FS-33),
Lane 18: 98-89 (1st), ***Lane 19:*** 98-89 (2nd) (FS-34),
Lane 20: 98-90 (1st), ***Lane 21:*** 98-90 (2nd) (FS-35),
Lane 22: ϕX 174 HinfI

2.3.2 t (8;21) (q22;q22)

For the 1st PCR reaction, GA-136 / GA 137 pair was used together with the *β-actin* control primers. In the second step, this reaction mixture was used as the template and primed with inner primers which is GA-138 / GA-139 pair. Figure 23 is the schematic diagram of the t (8;21) specific *AML-1 / ETO* fusion transcript, the location of the primers used for the RT-PCR analysis and the size of the PCR products generated. The sequence of the primers are given in Table 12.

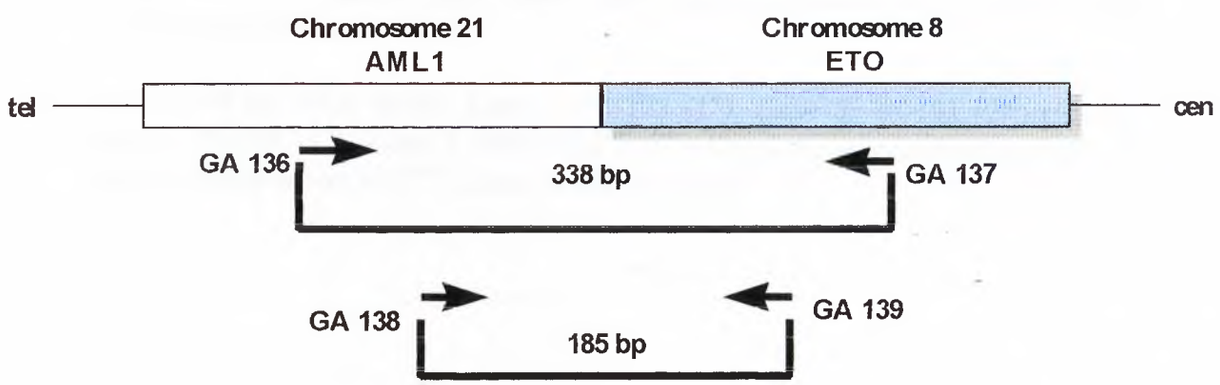

Figure 23: Schematic Representation of the Amplified Region for t (8;21)

(Adapted from Downing *et al.*, 1993)

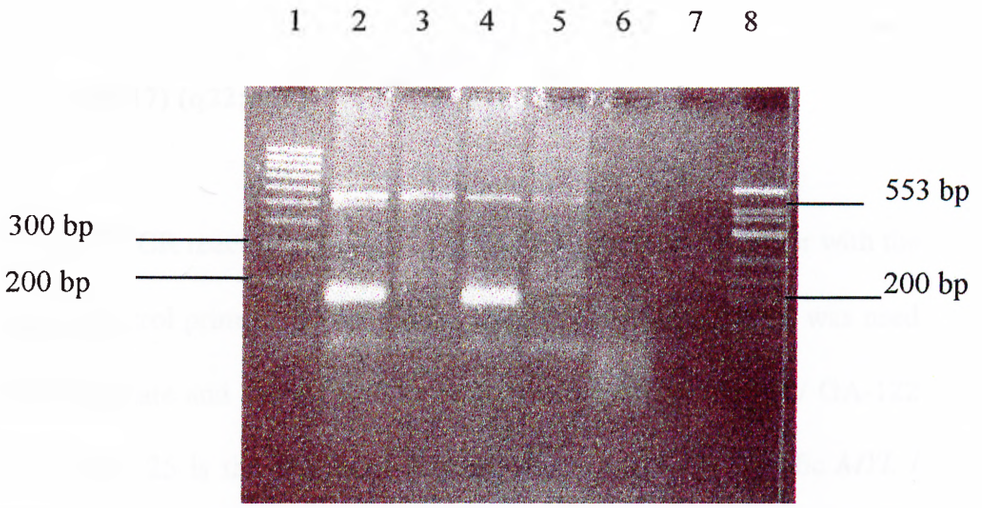

Figure 24 : RT-PCR Amplification for t (8;21) (q22;q22)

Lanes 2, and 4 indicate 1st PCR reactions while Lanes 3, and 5 indicate 2nd PCR reactions of the corresponding samples. Although PCR is successful, both of the samples, 98-123, and 98-124 are negative for the expected 185 bp fusion transcript (Lane3, Lane 5). Lane 6, and Lane 7 are negative controls of 1st and 2nd PCR reactions respectively.

Lane 1:100 bp DNA ladder, ***Lane 2:*** 98-123 (1st), ***Lane 3:*** 98-123 (2nd) (FS-46), ***Lane 4:*** 98-124 (1st), ***Lane 5:*** 98-124 (2nd) (FS-47), ***Lane 6:*** negative control (1st), ***Lane 7:*** negative control (2nd), ***Lane 8:*** ϕX 174 HinfI

2.3.3 t (15;17) (q22;q21)

For the 1st PCR reaction, GA-119 / GA 121 pair was used together with the *β-actin* control primers. In the second step, this reaction mixture was used as the template and primed with inner primers which is GA-120 / GA-122 pair. Figure 25 is the schematic diagram of the t (15;17) specific *MYL* / *RAR-α* fusion transcript, the location of the primers used for the RT-PCR analysis and the size of the PCR products generated. The sequence of the primers are given in Table 12.

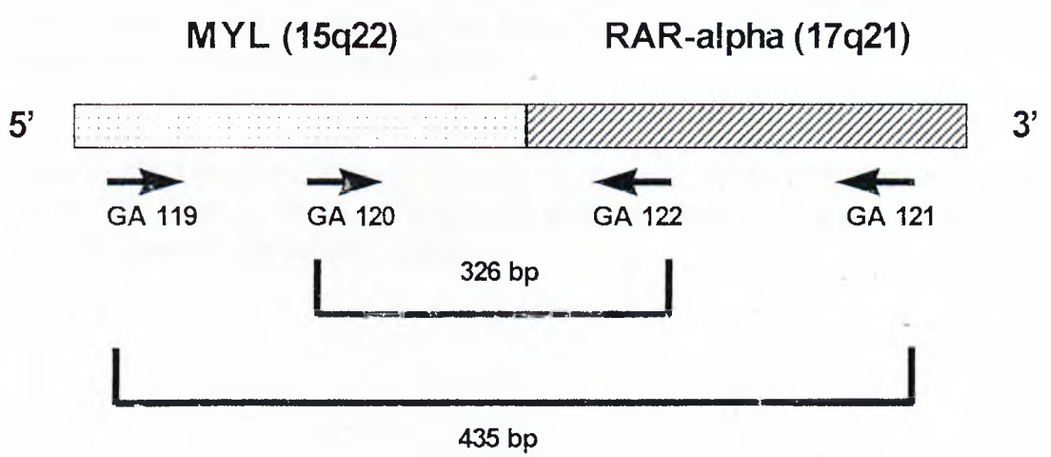

Figure 25: Schematic Representation of the Amplified Region for t (15;17)

(Adapted from Biondi *et al.*, 1992)

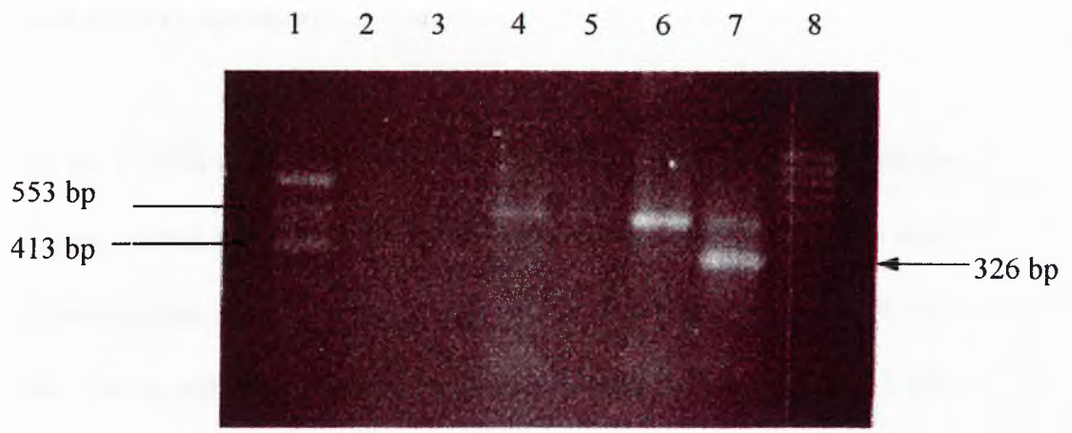

Figure 26: RT-PCR Amplification for t (15;17) (q22;q21)

Lanes 2, 4, and 6 indicate 1st PCR reactions while Lanes 3, 5, and 7 indicate 2nd PCR reactions of the corresponding samples. Sample 98-67 (FS-23) was observed to be positive for the 326 bp fusion transcript (see also Table 19) while the other sample, 98-56 (FS-23), was negative for the fusion transcript. PCR did not work for the sample 98-55 (FS-15) (Lanes 2, and 3).

Lane 1: ϕX 174 HinfI, ***Lane 2:*** 98-55 (1st), ***Lane 3:*** 98-55 (2nd) (FS-15), ***Lane 4:*** 98-56 (1st), ***Lane 5:*** 98-56 (2nd) (FS-15), ***Lane 6:*** 98-67 (1st), ***Lane 7:*** 98-67 (2nd) (FS-23), ***Lane 8: 100 bp DNA*** ladder

2.3.4 t (4;11) (q21;q23)

For the 1st PCR reaction, GA-134 / GA 135 pair was used together with the *β-actin* control primers. In the second step, this reaction mixture was used as the template and primed with inner primers which is GA-132 / GA-135 pair. For an additional *ALL-1* gene internal control, GA-132 / GA-133 pair was also used. Figure 27 is the schematic diagram of the t (4;11) specific *ALL-1 / AF-4* fusion transcripts. The location of the primers used for the RT-PCR analysis and the size of all possible PCR products generated according to breakpoint positions and alternative splicing on *ALL-1 and AF-4* genes are also depicted.

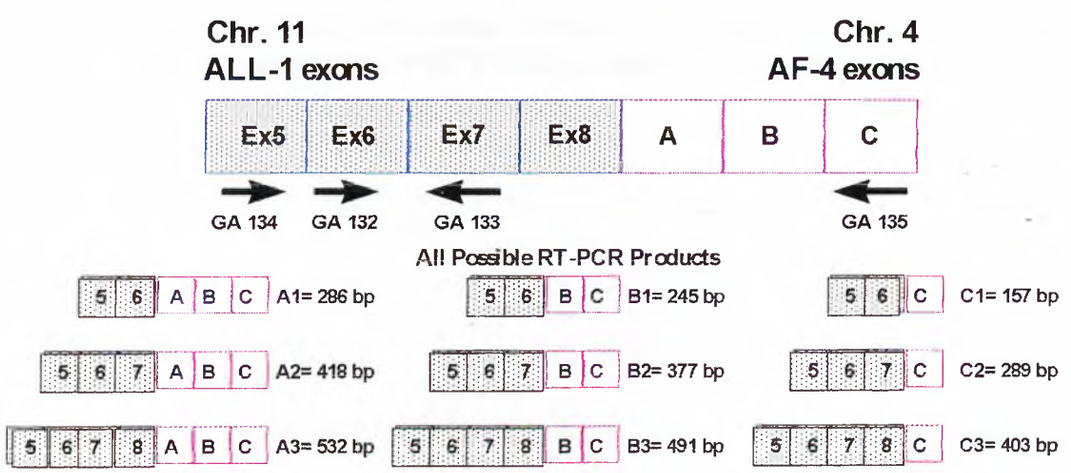

Figure 27: Schematic Representation of the Amplified Region for t (4;11)

(Adapted from Cimino *et al.*, 1996)

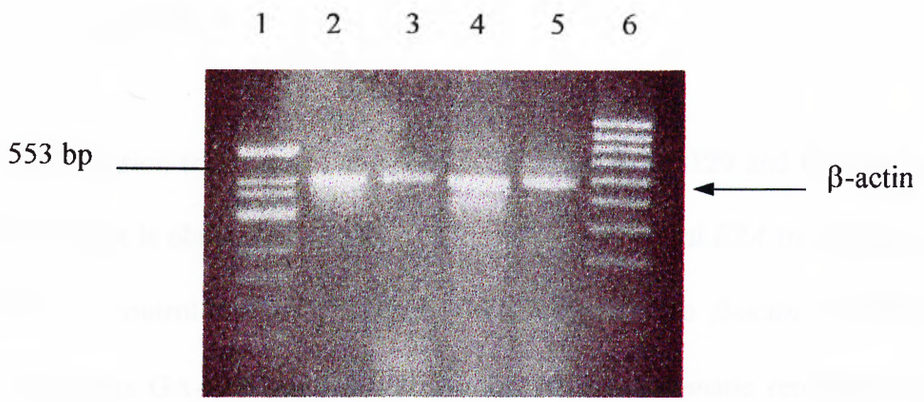

Figure 28 : RT-PCR Amplification for t (4;11) (q21;q23)

Lanes 2, and 4 indicate 1st PCR reactions while Lanes 3, and 5 indicate 2nd PCR reactions of the corresponding samples. Both of the samples, 98-29 (FS-3) and 98-34 (FS-5) were negative for t (4;11) specific transcripts even though *β-actin* was successfully amplified.

Lane 1: ϕX 174 HinfI, ***Lane 2:*** 98-29 (1st), ***Lane 3:*** 98-29 (2nd) (FS-3), ***Lane 4:*** 98-34 (1st), ***Lane 5:*** 98-34 (2nd) (FS-5), ***Lane 6:*** 100 bp DNA ladder

2.3.5 t (1;19) (q23;p23)

E2A / PBX-1 fusion transcript is amplified with primers GA-129 and GA-130 and a 164 bp transcript is obtained. A 180 bp fragment of the normal *E2A* transcript is also amplified as a control for RNA integrity, in addition to the *β-actin* amplification, using the primers GA-129 and GA-131. Figure 29 is a schematic representation of PCR products obtained together with the primers used for RT-PCR analysis.

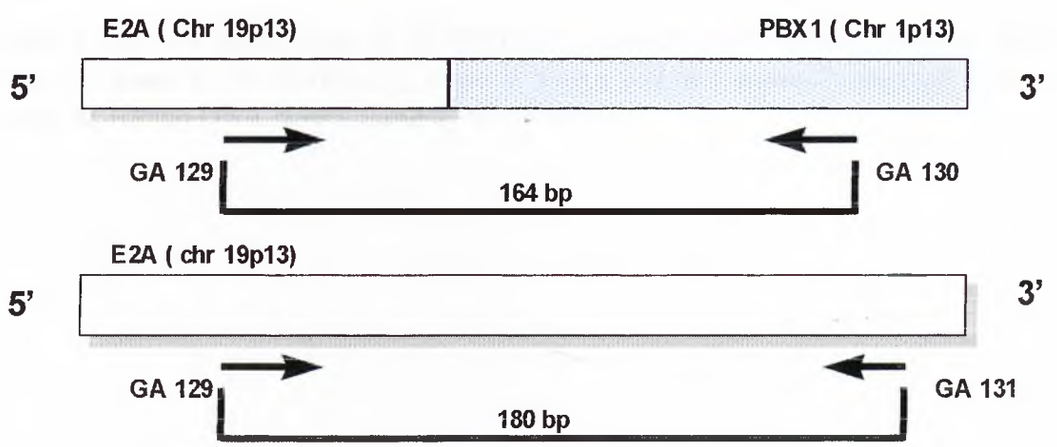

Figure 29: Schematic Representation of the Amplified Region for t (1;19)

(Adapted from Privitera *et al.*, 1992)

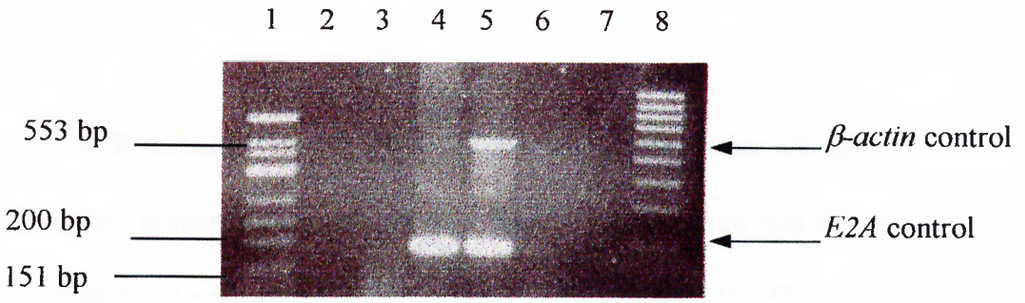

Figure 30: **RT-PCR Amplification for t (1;19) (q23;p23)**

For samples 98-29 (FS-3) (Lane 2), 98-35 (FS-6) (Lane 3), and Focus cell line (translocation negative) (Lane 6), PCR did not work. For 98-48 (FS-11) (Lane 4), and 98-52 (FS-12) (Lane 5), control reaction products were present but no t (1;19) specific fusion transcripts could be detected.

Lane 1: ϕX 174 HinfI, ***Lane 2:*** 98-29 (FS-3), ***Lane 3:*** 98-35 (FS-6), ***Lane 4:*** 98-48 (FS-11), ***Lane 5:*** 98-52 (FS-12), ***Lane 6:*** Focus cell line, ***Lane 7***: negative control, ***Lane 8:*** 100 bp DNA ladder ***Lane 2:*** 98-29 (FS-3)

2.3.6 inv 16 (p13;q22)

For the 1st PCR reaction, GA-128 / GA 125 pair was used together with the *β-actin* control primers In the second step, this reaction mixture was used as the template and primed with inner primers which is GA-126 / GA-127 pair. The most common outcome is a 414 bp 1st PCR product and 221 bp 2nd PCR product. Figure 31 is the schematic diagram of the inv 16 specific *CBFB / MYH11* fusion transcripts (A, B, C, D). The location of the primers used for the RT-PCR analysis and the size of all possible PCR products generated are also depicted.

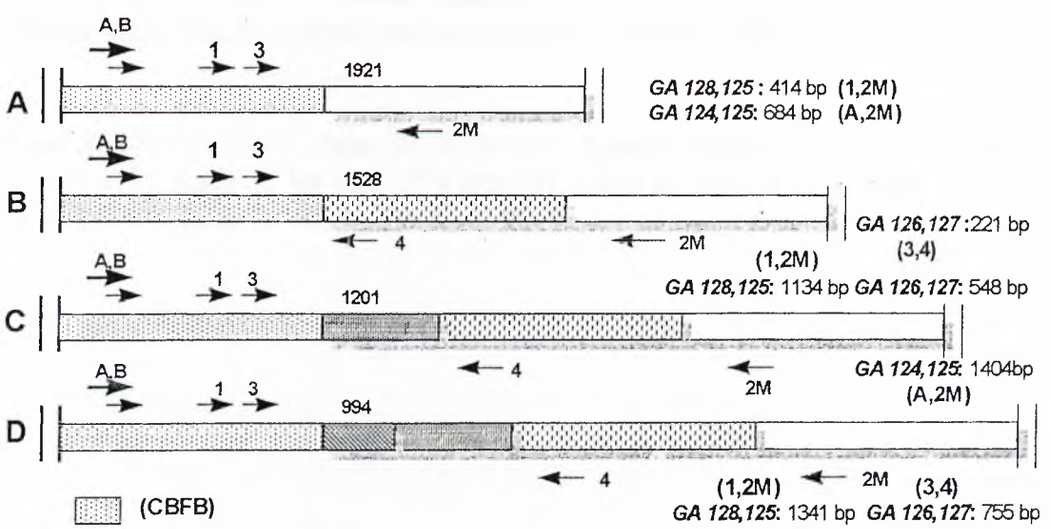

Figure 31: Schematic Representation of the Amplified Region for inv 16

(Adapted from Claxton *et al.*, 1994)

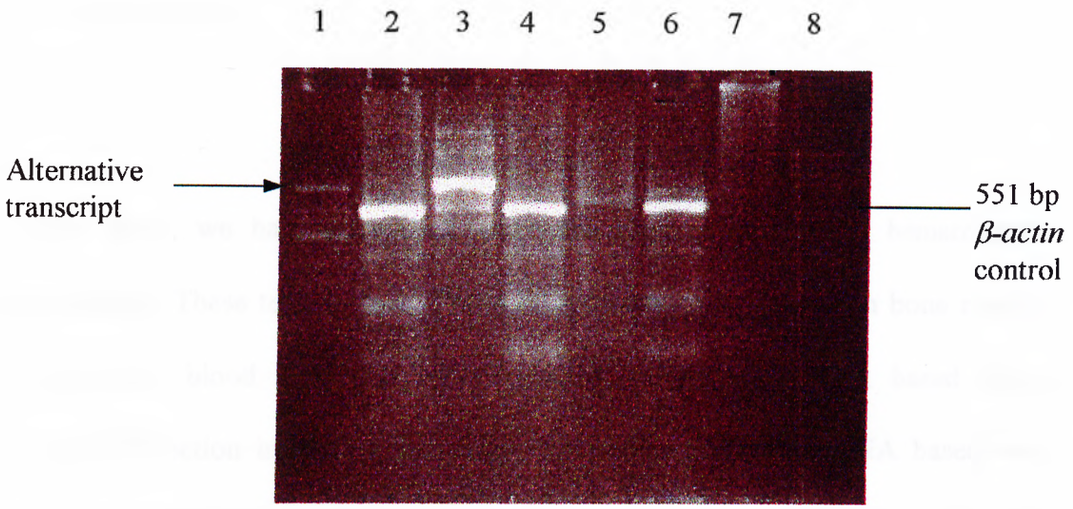

Figure 32: RT- PCR Amplification for inv 16 (p13;q22)

Lanes 2, 4, and 6 indicate 1st PCR reactions while Lanes 3, 5, and 7 indicate 2nd PCR reactions of the corresponding samples. Sample 98-84 (FS-29) was observed to have a strong band at around 800 bp level which is unique to 2nd PCR (Lane 3). This might be an indication of an alternatively spliced transcript. For the other samples 98-123, and 98-124 the inv 16 fusion transcripts were not detected (Lanes 5, and 7 respectively). *β-actin* control was successfully amplified (Lanes 2, 4, and 6).

Lane 1: ϕX 174 HinfI, ***Lane 2:*** 98-84 (1st), ***Lane 3:*** 98-84 (2nd) (FS-29), ***Lane 4:*** 98-123 (1st), ***Lane 5:*** 98-123 (2nd) (FS-46), ***Lane 6:*** 98-124 (1st), ***Lane 7:*** 98-124 (2nd) (FS-47), ***Lane 8:*** 100 bp DNA ladder

IV. DISCUSSION

In this study, we have introduced molecular diagnostic tests for hematological malignancies. These tests include DNA based chimerism evaluation in bone marrow or peripheral blood stem cell transplantation patients; and RNA based fusion transcript detection in various hematological malignancies. The DNA based tests relied on the analysis of four STR (vWA, THO1, F13A01, and HUMFES / FPS) and one VNTR (PAH) polymorphisms. The RNA based tests targeted six frequently seen chromosomal rearrangements associated with hematological malignancies. These are t (9;22), t (15;17), t (8;21), t (4;11), t (1;19), and inv 16. To the best of our knowledge, this study presents the first molecular genetic data on hematological malignancies associated chromosomal rearrangements in the Turkish population with the exception of t (9;22) which was done previously (Eren *et al.*, 1996).

The first part of this study involves the evaluation of chimerism status in patients who underwent allogeneic transplantation. Due to the fact that the degree of chimerism achieved after transplantation has strong influence on the outcome of transplantation, in development of GVHD, in failure or graft rejection, or in relapse of disease; chimerism evaluation is of great importance. Chimerism status is influenced by a number of factors such as gender, incidence of GVHD, disease type, conditioning regimen, and HLA compatibility. Detection of complete chimerism (CC) is a good prognostic factor in patients who underwent transplantation. On the other hand,

mixed chimerism has been correlated with leukemic relapse therefore, detection of mixed chimerism is useful in monitoring patients susceptible to leukemic relapse.

PCR-based analysis of DNA polymorphisms is a very sensitive technique and the sensitivity is further increased by the use of highly polymorphic loci. Therefore an important factor in the DNA polymorphism based chimerism analysis is the choice of polymorphic loci. One VNTR and four STR loci were analyzed in 22 recipient-donor pairs. Twelve patients (54.5 %) were complete chimeric, and one patient was mixed chimeric. A total of six cases were non- informative for all the loci analyzed. There were three rejection cases two of which had β-thalassemia and one had CML. It is known that if relapse is demonstrated for CML patients, IFN-α is a therapy of choice when another transplantation is not available. This patient has been demonstrated to have rejection at a relatively early post-transplantation period (+140) (Table 17, KIT-30). Therefore, alternative therapy options may well be considered. For the non-informative cases, definitely, different polymorphic loci should be included for the analysis.

Molecular monitoring of transplantation patients is invaluable in the early detection and confirmation of relapse. We have experienced this fact in one of our acute myelogenous leukemia patients. KIT-4 is an AML-M4 patient who was in hematologic complete remission when she was reported to be complete chimeric based on our molecular analysis as well. The analysis was performed with all four STR loci at first and she was found to be informative at vWA locus with which

further analyses were carried out. After a few months she relapsed with a different type of leukemia, namely plasma cell leukemia. STR analysis of her peripheral blood sample and bone marrow aspirate revealed mixed chimerism confirming the relapse at molecular level. Then the patient was in second complete remission by the chemotherapy and DLI (donor lymphocyte infusion) and complete chimerism was shown for the second time by the examination of peripheral blood cells and bone marrow. Five months later, she had a second relapse with blastic cells in CSF (cerebrospinal fluid). STR analysis was performed by examining both the peripheral blood cells and also CSF of the patient. The peripheral blood smear and bone marrow aspirate revealed that she was still in second complete remission and genotype results were compatible with complete chimerism. However, although CC was the case in peripheral blood cells, STR analysis of CSF showed mixed chimerism which was consistent with the CNS (central nervous system) confined relapse. This case was important in two aspects. The first one is that, repetitive chimerism analyses is of vital importance for close monitoring of the disease and the for the detection of the time of relapse. The second one is that, PCR-based chimerism evaluation is sensitive enough to confirm the clinical and hematological situation of patients with such malignancies. It is known that isolated CNS relapse occurs rarely in patients treated with bone marrow transplantation and the finding that the patient was complete chimeric in her peripheral blood cells but mixed chimeric in her CSF was consistent with the clinical findings of CNS relapse without systemic relapse.

Among the 22 recipient-donor pairs studied, THO1 was informative in ten of 22 (45.5 %) pairs, F13A01 in five of 22 (22.7 %), vWA in four of 22 pairs (18.2 %), and FES / FPS in three of 22 pairs (13.6 %). In ten pairs PAH VNTR was also analyzed and was found to be informative in two of 10 (20 %) pairs. Six of these ten pairs were the ones that were non-informative with the four STR loci. They were still non-informative after PAH VNTR analysis was also included (Table 17).

Regarding the time of PCR analysis, complete chimerism could be detected 15 days post-transplantation as the earliest, and 2920 days (about 8 years) post-transplantation as the latest which is quite significant. The latest duration is an indicative of how long the complete chimerism status is maintained and thus is a good prognostic factor. These repetitive analyses not only enables the detection of the time of relapse but also provides the physician with the information on whether to repeat the transplantation procedure or continue with alternative follow-up approaches.

In conclusion, PCR-based detection of DNA polymorphisms appears to be a sensitive and powerful technique for the evaluation of chimerism status in patients treated with allogeneic transplantation. The availability of different polymorphic marker will increase the sensitivity of the analysis. The evaluation of the prognostic value and clinical significance of the DNA polymorphism results require further analysis of more patients for longer periods. A final remark is that this system is established and is applicable for prognostic analyses.

The second part of this study focused on the detection of fusion transcripts that are generated by chromosomal rearrangements in various acute and chronic leukemias. This approach has both diagnostic and prognostic implications depending on whether the patient has undergone any kind of treatment or not. It is diagnostically important because, certain chromosomal rearrangements are closely associated with certain types and even subtypes of hematological malignancies. In this respect, the detection of fusion transcripts not only adds accuracy to the diagnosis brought by the conventional means but also provides insight into the pathogenesis of the malignancy under question. This approach has also prognostic importance because it provides the detection of minimal residual disease for the patients who have undergone allogeneic transplantation. MRD is an indicator for a prediction of relapse. MRD negativity at the termination of therapy, which could be demonstrated by the RT-PCR based analyses, suggests long term DFS (disease free survival). Therefore, more precise evaluation of MRD is necessary with regard to therapeutic strategy in monitoring of the disease. RT-PCR based analysis of the chromosomal breakpoints and the detection of fusion transcripts is a very sensitive and powerful approach for this evaluation.

Due to the importance of such an evaluation, special precautions are needed to avoid false positive results due to contaminants and false negative results due to insufficient yields. To avoid false positives, two kinds of negative controls, a normal translocation negative RNA sample, and a blank sample containing all the required reagents except for RNA, were used. For the normal RNA sample, translocation negative

hepatocellular carcinoma cell lines (Focus, WRL, HT29) were used. cDNA samples synthesized from the RNA obtained from those cell lines were amplified with translocation specific primers and no amplification was observed with any of the rearrangements analyzed in the panel (Table 15). To eliminate false negatives resulting from RNA degradation, poor reverse transcription or inadequate PCR amplification, cDNA fragments spanning the *β-actin* gene and / or one of the genes that are disrupted due to translocations (*c-ABL* in t(9;22) Figure 21, *ALL-1* in t(4;11) Figure 27, and *E2A* in t(1;19) Figure 29) were coamplified together with the translocation specific fusion transcripts. The absence of *β-actin, c-ABL, ALL-1* or *E2A* cDNAs would indicate false negativity. These precautions were kept throughout the analyses.

The translocation t (9;22) (q34;q11), which is typical for CML (95 %), but is also seen in AML and ALL, generates two kinds of fusion transcripts (198 bp, and 273 bp) depending on the actual breakpoint in *BCR*. 198 bp transcript is the product when the breakpoint is in the first intron. This analysis could help in diagnosis to discriminate between a lymphoid blast crisis of CML and *de novo* Ph+ bcr- ALL for the patients who are newly diagnosed. This discrimination is difficult because both leukemic states are clinically similar with extremely poor prognosis and the similar blast morphology of leukemic cells (Hermans et al., 1987)

This rearrangement was analyzed in CML (n=26), AML (n=13), MDS (n=3), and ALL (n=1) patients. Among the CML cases eight (FS-10, FS-16, FS-27, FS-31, FS-33, FS-35, FS-38, and FS-45) were newly diagnosed and did not have any treatment.

Except FS-27 and FS-38, the other five patients are translocation positive (Table 20). Three of the patients (FS-17, FS-20 and FS-43) were under interferon treatment and were shown to have the 198 bp transcript. Eight patients have been reported to undergo allogeneic BMT between the years 1992-1998. When the results are examined, it is seen that all of these eight patients were negative for both of the t(9;22) transcript at the time of RT-PCR analysis. This supports that BMT is the treatment of choice for CML patients. In none of the analyzed AML, ALL and MDS patients t (9;22) transcripts were observed. This was not unusual because of the strong association between CML and the presence of Ph chromosome.

The translocation t (8;21) (q22;q22) is seen in 20 % of AML-M2 cases and is associated with good prognosis. This rearrangement is analyzed in 13 AML cases with various subtypes (Table 19). The subtypes of two patients (FS-18, FS-47) are unknown and one patient (FS-14) has biphenotypic AML. The analysis was also done for three MDS patients (FS-5, FS-6, FS-12) as well. All of these patients were negative for t (8;21) fusion transcript.

The t (15; 17) (q22; q21) is unique to AML-M3 (70-100 %) with intermediate prognosis. The detection of PML / RAR-α transcript has important implications not only for assessing the MRD status, but also for management of patients with APL. It is a useful marker for APL diagnosis to identify patients who can benefit from retinoic acid treatment. In this context, this rearrangement was analyzed in 13 AML and three MDS patients. The single positive result belongs to one AML-M4 patient (FS-23).

The inv 16 (p13;q22) rearrangement is observed in 25 % of AML-M4 cases with good prognosis. The analysis was performed on 14 AML patients and three MDS patients. Altered size fragments were observed for two cases (FS-8, FS-29).These findings suggest that altered size fragments are alternative splicing products. Sequencing of the 2nd PCR products of these samples would be suggested to determine the actual breakpoints in the differentially spliced *MYH11* gene. Another important observation was that, the expected 221 bp product was observed in two patients with M4 subtype (FS-2, and FS-21). FS-2 has been reported to have relapse which is consistent with the presence of MRD.

The rearrangements t(4;11) (q21;q23) and t(1;19) (q23;p23) are observed in 100 % lymphoblastic ALL and 20-25 % pre-B cell ALL. In this study, these rearrangements are analyzed in three MDS and one ALL cases. However, in none of them the related transcripts could be observed. This is most probably due to the small size of the sample group. All necessary precautions were taken to avoid false negatives.

We have successfully demonstrated the presence of t (9;22), t (15;17), and inv 16 in our patient population. A more comprehensive study including cytogenetic analysis, and sequencing is needed together with fusion transcript detection to further characterize the altered size transcripts observed particularly in inv 16. They most probably represent alternative chromosomal breakpoints. In addition, all the results that have been reported as positive might be confirmed with Southern blot analysis using the fusion region specific probes for the hybridization.

In conclusion, the importance of molecular analyses in hematological malignancies comes from the fact that, they serve not only to detect but also to prevent, treat and reinduce remission in patients that relapsed after the allogeneic transplantation. These analyses should be performed widespread with a collaborative and comprehensive understanding to help patients with hematological malignancies.

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