ANALYSIS OF CANDIDATE MOLECULAR TARGETS IN ADULT (CML) AND

CHILDHOOD (AML, ALL) LEUKEMIAS

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By

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May, 2004

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

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ABSTRACT

ANALYSIS OF CANDIDATE MOLECULAR TARGETS IN ADULT (CML) AND CHILDHOOD (AML, ALL) LEUKEMIAS

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Ph.D. in Molecular Biology and Genetics
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Candidate molecular targets were investigated in three different types of leukemias, chronic myeloid leukemia (CML), acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL). The first group of these molecular targets was identified through a cDNA based gene expression profile analysis in sixty-seven CML patients who were classified according to clinical parameters known as new prognostic score (NPS). CML patients can be divided into three groups of low-risk, intermediate-risk, and high-risk, based on NPS. Response of these risk groups to treatment is not uniform and the gene expression profiles associated with each risk group remain unknown. Seven genes were chosen from a cDNA microarray study in which two high versus two low-risk patients were analyzed. Semi-quantitative and real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of these differentially expressed transcripts highly correlated with the microarray data. Expression levels of all genes, except *PTGS1*, were significantly different between the high (n=9) and low-risk (n=7) CML by semi-quantitative RT-PCR (*IFITM1* and *CXCL3* p=0.001; *CCNH* p=0.012; *RAB1A* p=0.01, *PRKAR2B* p=0.016; *UCP2* p=0.04; and *PTGS1* p=0.315). Real-time RT-PCR analysis showed similar results for *IFITM1* expression in thirty-four low and eleven high-risk patients (p=9.7976 x 10^{-11}). Higher *IFITM1* or lower *CXCL3* expression correlated with improved survival (p=0.01 and p=0.059 respectively). Gene expression profiling is a valuable tool to identify candidate risk group indicator genes for the development of a molecular classification system for CML, which may also predict survival.

Although the connection between DNA-repair gene mutations and hematological malignancies are now well established, germ-line mutations in the base excision repair (BER) pathway was only recently documented in an inherited cancer syndrome in human homologue of *E. coli mut Y* (*MYH*). Interestingly, the cancer associated *MYH* missense mutations Tyr165Cys and Gly382Asp have been documented with a high frequency (1 percent) in a control group of the British population. Therefore, we screened the above mentioned missense variants in two different childhood leukemias, AML (n=45) and ALL (n=140). Neither mutation was present in any of the patient samples and controls, except for one patient diagnosed with AML/M3. Tyr165Cys mutation in the heterozygous state was present in the sample obtained at the time of initial diagnosis. Further sampling, at remission, and the analysis of parental DNA, showed only the normal allele. Therefore, the mutation was considered to be specific for the leukemic blasts. Based on these results, an association between childhood leukemias and the *MYH* missense variants Tyr165Cys and Gly382Asp was not observed. Also, these variants appear to be absent -if not at a very low frequency- in the Turkish population, contrary to the British population.

ÖZET

ADAY MOLEKÜLER HEDEFLERİN YETİŞKİN (KML) VE ÇOCUKLUK ÇAĞI (AML, ALL) LÖSEMİLERİNDE İNCELENMESİ

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Aday moleküler hedefler üç farklı lösemi tipinde incelenmiştir. Bunlar, kronik miyelositer lösemi (KML), akut miyelositer lösemi (AML) ve akut lenfositik lösemi (ALL)' dir. İlk moleküler hedef grubu yeni prognostik skorla sınıflandırılmış altmış yedi KML hastasında cDNA'ya bağlı gen ifade profillerinin incelenmesi ile saptanmıştır. Hastalar, yeni skorlama ile yüksek, orta ve düşük riskli olarak sınıflandırılmıştır. Risk gruplarının tedaviye cevapları farklıdır ve her grubun gen ifade profilleri bilinmemektedir. cDNA mikrodizilimleri kullanılarak iki yüksek ve iki düşük riskli hasta karşılaştırılmış ve yedi adet gen seçilmiştir. Yarı-nicel ve gerçek zamanlı ters yazılımlı polimeraz zincir reaksiyonu (RT-PCR) yapılmış ve sonuçların cDNA mikrodizilimleri ile benzer olduğu gösterilmiştir. *PTGS1* dışındaki tüm genlerin ifadeleri, yarı-nicel RT-PCR sonucuna göre, istatistiksel olarak yüksek (n=9) ve düşük (n=7) riskli grup arasında anlamlı farklılık göstermektedir (*IFITM1* ve *CXCL3* p=0.001; *CCNH* p=0.012; *RAB1A* p=0.01, *PRKAR2B* p=0.016; *UCP2* p=0.04; ve *PTGS1* p=0.315). Gerçek zamanlı RT-PCR düşük (n=34) ve yüksek (n=11) riskli hastalarda *IFITM1* için benzer sonuçları vermiştir (p=9.7976 x 10^{-11}). Kaplan-Meier analizleri sonucunda yüksek *IFITM1* veya düşük *CXCL3* ifadelerinin sağ kalımla ilişkili olduğu saptanmıştır (sırası ile p=0.01 ve p=0.059). Sonuçlarımız, gen ifade profillerinin risk gruplarının tanımlanmasında kullanılabileceğini göstermekte ve ayrıca sağ kalımları belirlemekte yardımcı olabilmektedir.

DNA onarım genlerindeki mutasyonlarla hematolojik hastalıklar arasındaki ilişki iyi bilinmekle beraber, baz çıkarma onarım genlerinden insan *E. coli mut Y* homoloğu *MYH*'deki eşey hücre mutasyonu kalıtsal bir kanser hastalığında ilk kez geçtiğimiz yıl gösterilmiştir. Kanserle ilgili *MYH* mutasyonlarından Tyr165Cys ve Gly382Asp İngiliz kontrol populasyonunda yüksek sıklıkta (yüzde 1) gözlenmiştir. Bu bulgulara dayanarak, çocukluk çağı lösemileri olan AML (n=45) ve ALL (n=140) hastalarında yukarıda belirtilen mutasyonları kandan elde edilen DNA molekülünde inceledik. Mutasyonlar AML/M3 tanısı olan bir hasta hariç, diğer hasta örneklerinde ve kontrolde saptanmamıştır. Tyr165Cys mutasyonu tanı sırasında alınan örnekte heterozigot olarak bulunmaktaydı. Remisyonda alınan örnek ve ebeveyn DNA'ları incelendiğinde sadece normal alel gözlenmiştir. Bu nedenle, mutasyonun lösemik blastlara özgü olduğu düşünülmüştür. Bu sonuçlar çocukluk çağı lösemileri ile *MYH* mutasyonları Tyr165Cys ve Gly382Asp arasında bir ilşki bulunabileceğini göstermemiştir. Son olarak, bu mutasyonların kontrol grubunda gösterilememesi Türk toplumundan elde edilen sonuçların İngiliz toplumundan farklı olduğunu düşündürmüştür.

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ABBREVIATIONS

A	absorbance
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
ATP	adenine triphosphate
bp	base pair
BSA	bovine serum albumin
BER	base excision repair
cAMP	cyclic adenosine mono phosphate
cDNA	complementary DNA
CML	chronic myeloid leukemia
C-terminus	carboxy terminus
Cy3	indocarbocyanine
Cy5	indodicarbocyanine
dATP	adenosine deoxyribonucleoside triphosphate
dCTP	cytosine deoxyribonucleoside triphosphate
ddH$_2$O	deionized water
ddNTP	dideoxynucleotide triphosphate
DEPC	diethylpyrocarbonate
dGTP	guanosine deoxyribonucleoside triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triposphate
dTTP	thymine deoxyribonucleoside triphosphate
DTT	dithiothretiol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	gram
IVT	in vitro transcription
kb	kilobase
LB	Luria-Bertani medium
LOH	loss of heterozygosity
LOI	loss of imprinting
M	molar
min	minute
ml	milliliter
mm	milimeter
mM	milimolar
μl	microliter
mRNA	messengerRNA

NaOAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hyrdoxide
ng	nanogram
nm	nanometer
NPS	new prognostic score
N-terminus	amino terminus
OD	optical density
Oligo(dT)	oligodeoxythymidylic acid
O/N	over night
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
RNA	ribonucleic acid
rpm	revolution per minute
SDS	sodium dodecyl sulphate
sec	second
TAE	tris-acetic acid-EDTA
TBE	tris-boric acid-EDTA
Tris	Tris (hydroxymethyl)-methylamine
U	unit
UV	ultraviolet
v	volt
v/v	volume for volume
μg	microgram
μl	microliter

CHAPTER 1. INTRODUCTION

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).

In 1924, Maximow 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 has the ability of self-renewal and the production of progeny destined to differentiate (Lee *et al.,* 1993). The common pluripotent hematopoietic stem cell (PHSC) gives rise to lymphoid and the trilineage myeloid stem cells (CFU-S) (Figure 1) (Cotran *et al.,* 1989).

The lymphoid stem cell (LSC) 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, morphological recognizable stages in lymphoid differentiation. From the multipotent myeloid stem cell, three

types of committed stem cell arise which differentiate along the erythroid / megakaryocytic, eosinophilic, and granulocyte / macrophage pathways (Cotran *et al.,* 1989). 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 Figure 1, granulocytes and macrophages have a common precursor, CFU-GM. 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 primitive one. The later stage is CFU-E. From all these different committed stem cells, intermediate stages are derived, and finally the morphological 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).
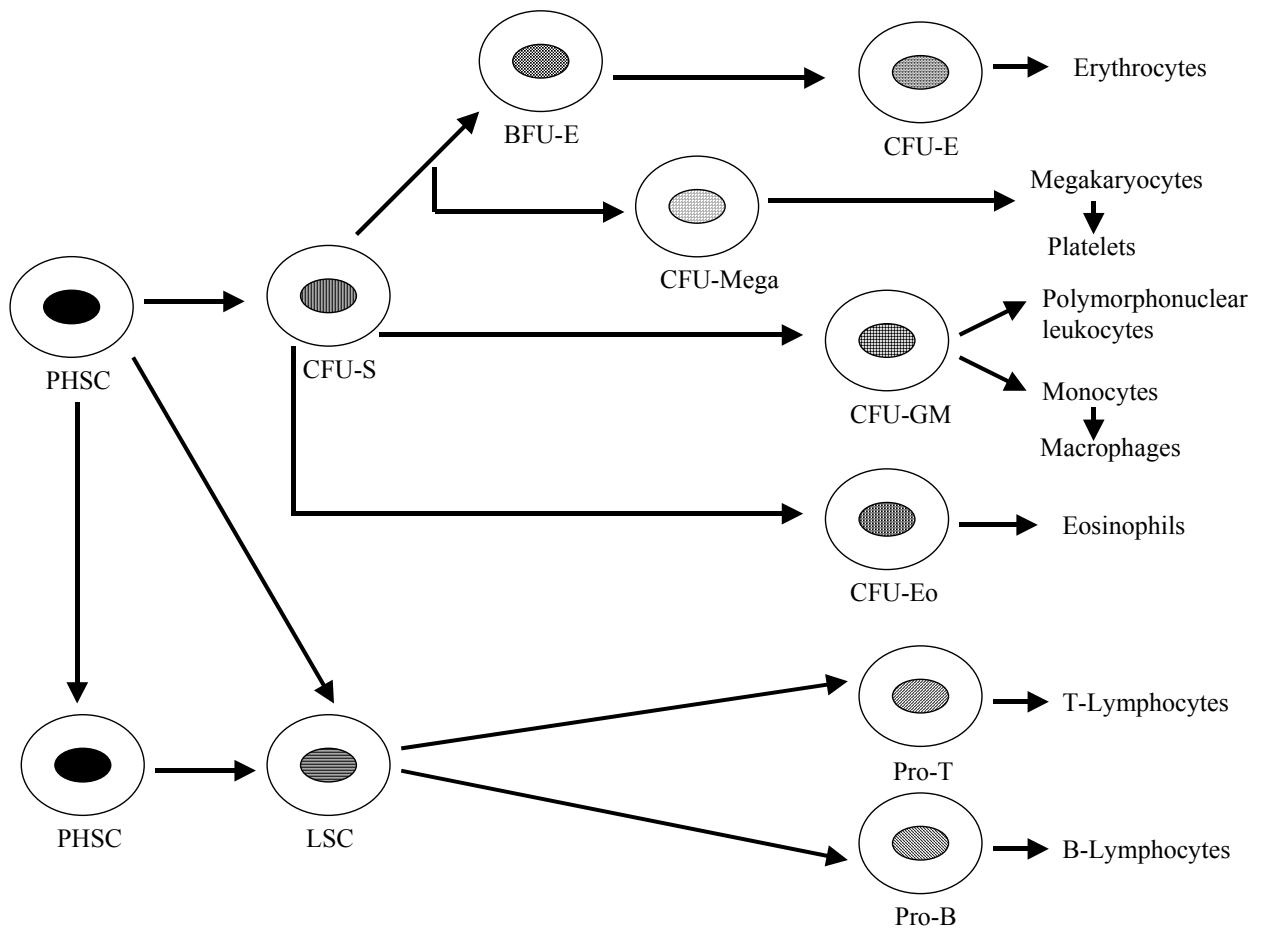
Figure 1: Differentiation of hematopoietic cells (adapted from Cotran *et al.,* 1989).

1.2 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 the 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. 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)

1.3 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. 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 arises in immature hematopoietic cells. They are characterized by the disturbance of normal hematopoiesis and 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. Leukemias account approximately 5% of all cancers (http://www.iarc.fr).

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, cancer chemotherapy, radiation, and viruses such as human T-cell lymphotropic virus type-I (HTLV-I) in adult T-cell leukemia/ lymphoma are among the well-established leukomogenic factors (Wilson *et al.,* 1991). 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 or a syndrome of chromosomal instability (Wilson *et al.,* 1991).

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. However, some adult acute leukemias are "biphenotypic" in nature, with the expression of both lymphoid and myeloid linage cell surface antigens. In addition, in a subset of acute leukemias, lymphoid and 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).

The classification of leukemias is based on the cell type involved and the state of maturity of the leukemic cells. Thus, acute leukemias are characterized by the presence of very immature cells, blasts, and 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 known as lymphocytic and myelocytic. Therefore, a simple classification yields four patterns of leukemia: acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelocytic leukemia (AML) and chronic myelocytic (myeloid) leukemia (CML) (Cotran *et al.,* 1989).

Most commonly leukemias are diagnosed by a blood test to count the number of red cells, white cells and platelets. A biopsy of the bone marrow may also be performed.

1.4 Chronic myeloid leukemia (CML)

1.4.1 Epidemiology and clinical characteristics of CML

CML is a clonal myeloproliferative disease that results from neoplastic transformation of primitive hematopoietic progenitor cells. It involves myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid and occasionally T-lymphoid lineages (Cortes *et al.,* 1996; Faderl *et al.,* 1999a; Kabarowski *et al.,* 2000).

CML accounts for 7-15% of leukemias in adults, and it has an incidence of 1-2 cases per 100,000 people per year (Cortes *et al.,* 1996; Faderl *et al.,* 1999a; Faderl *et al.,* 1999b). The median age at presentation is 45-55 years, but the disease can be seen in all age groups. The etiology of the disease is not clear. 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 is historically important in two aspects. It was the first human disease, in which a specific abnormality of the karyotype (Philadelphia chromosome (Ph)) could be linked to pathogenetic events of leukemogenesis (Faderl *et al.,* 1999a; Deininger *et al.,* 2000a). Second is at the therapeutic level. CML is one of the first neoplastic diseases in which the use of a biological agent interferon could suppress the neoplastic clone and prolong survival (Faderl *et al.,* 1999a).

The disease is clinically divided into three phases as chronic, accelerated and blastic. The most of the cases (85%) are diagnosed at the chronic phase (CP) (Faderl *et al.,* 1999a; Chase *et al.,* 2001). In this phase, the number of myeloid progenitor cells increase and since differentiation continues, matured granulocytes are found in the peripheral blood. This period is approximately 3-5 years. The accelerated phase (AP) begins after several weeks to several years. Terminal differentiation is quickly lost; thrombocytosis, basophilia and multiple cytogenetic differences are added to the picture. The third phase known as blastic phase (blastic crisis (BC)) characterizes the last phase in which unmatured blast cells immediately increase. 30% or more leukemic cells are found in the peripheral blood or bone marrow. At this period, average survival is 3-6 months (Faderl *et al.,* 1999a).

CML patients can be divided into three groups of low-risk, intermediate-risk, and high-risk, based on clinical parameters (age, spleen size, blast, platelet, eosinophil and basophil counts) known as new prognostic score (NPS) (Hasford *et al.,* 1998; Bonifazi *et al.,* 2000). This scoring is the modified form of Sokal score, which worked well as a prognostic discriminator for survival of three different risk groups (Sokal *et al.,* 1984; Sokal *et al.,* 1985; Hehlmann *et al.,* 1997). Response of these risk groups to treatment is not uniform. Low risk patients respond better to treatment including interferon-alfa administration and their survival is much better compared to high-risk ones (Alimena *et al.,* 1996; Kloke *et al.,* 2000). The molecular characteristic of these three groups of patients is currently unknown.

1.4.2 Genetic and epigenetic factors in CML

Although CML was the first human disease in which a specific abnormality of the karyotype could be linked to pathogenetic events of leukemogenesis, genetic factors related to CML are not very well known. The specific chromosomal abnormality is known as Philadelphia chromosome (Ph) which results from reciprocal translocation of the long arms of chromosomes 9 and 22, t(9;22) (q34;q11). It is observed in 90-95% of all cases (Kurzrock *et al.,* 1988; Faderl *et al.,* 1999a; Warmuth *et al.,* 1999a).

. The translocation breakpoint encompasses *ABL* on 9q34 and *BCR* on 22q11. As a result of this translocation, different numbers of *BCR* exons are added to chimeric *BCR/ABL* fusion gene. The *BCR* sequences in this mRNA are responsible for converting cellular proto-oncogene *ABL* to an oncogene (Heisterkamp *et al.,* 1985). BCR causes ABL to become constitutively active as a tyrosine phosphokinase. The chimeric protein has higher tyrosine phosphokinase activity than the normal ABL protein (Faderl *et al.,* 1999a; Chopra *et al.,* 1999; Deininger *et al.,* 2000a; Deininger *et al.,* 2000b). The main characteristics of this disease can be listed as, adhesion independence, growth factor independence, and resistance to apoptosis (Di Bacco *et al.,* 2000).

Cytogenetic and molecular changes occur in 50-80% of patients during transition to accelerated and blastic phases (Faderl *et al.,* 1999a). Minor cytogenetic changes include monosomies of chromosomes 7, 17, and Y, trisomies of chromosomes 17 and 21, and translocation t(3;21)(q26;q22). Major changes include trisomies 8 and 19, isochromosome i(17q), and an extra Ph chromosome (double Ph) (Faderl *et al.,* 1999a). Trisomy 8 is the most common, and i(17q) occurs almost exclusively in the blastic phase. In addition molecular abnormalities in *p53*, *RB1*, *c-MYC*, *p16^{INK4A}*, *RAS* and AML-EVI-1, a fusion protein resulting from translocation t(3;21)(q26;q22) have been documented (Neubauer *et al.,* 1993; Faderl *et al.,* 1999a). Alteration of *p53* such as deletions, rearrangements, and mutations occur in 20-30% of patients with CML at blastic phase (Randhawa *et al.,* 1998; Faderl *et al.,* 1999a). Amplification of *c-myc* is found in 20% of patients (Randhawa *et al.,* 1998). Other events occurring more rarely include mutations in *RAS* (6%) and rearrangements and

deletions of *RB* (14%) and *p16* (15%) (Randhawa *et al.,* 1998). Majority of these structural aberrations are seen at the accelerated and blastic phases of the disease.

Interestingly, a novel type of genetic alteration in CML appears to be loss of imprinting (LOI), which has been demonstrated for insulin-like growth factor-II gene (*IGF2*). Genomic imprinting is an epigenetic modification of a specific parental allele of a gene, or the chromosome on which it resides, in the gamete or zygote leading to differential expression of the two alleles of the gene in somatic cells of the offspring. LOI is a new disease mechanism in cancer which involves loss of parental origin-specific expression of genes which leads to activation of the normally silent copy of growth-promoting genes and/or silencing of the normally transcribed copy of tumor suppressor genes. LOI of *IGF2* was documented in the accelerated and blastic phases of CML (Vogelstein *et al.,* 1998; Randhawa *et al.,* 1998).

1.4.3 Molecular biology of CML

1.4.3.1 Philadelphia chromosome (Ph)

The discovery of Ph chromosome in 1960 as the first consistent chromosomal abnormality associated with a specific type of leukemia was a breakthrough in cancer biology (Nowell *et al.,* 1960). It took 13 years before it was appreciated that the Ph chromosome is the result of a t(9;22) reciprocal chromosomal translocation (Rowley J.D., 1973) and another 10 years before the translocation was shown to involve the *ABL* proto-oncogene normally on chromosome 9 and a previously unknown gene on chromosome 22, later termed *BCR* for breakpoint cluster region (bcr) (Groffen *et al.,* 1984; Deininger *et al.,* 2000a). 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. 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 physiologic function of translocation partners

The *ABL* gene is the human homologue of the v-*abl* oncogene carried by the Abelson murine leukemia virus (A-MuLV) (Deininger *et al.,* 2000a). It is about 225 kb in size and is expressed as either 6 or 7 kb mRNA transcript, with alternatively spliced first exons, exon 1a and 1b respectively. The gene encodes a nonreceptor tyrosine kinase, which is higly conserved from *Drosophila* to humans (Laneuville P., 1995; Catherine *et al.,* 1998). This protein is a ubiquitously expressed 145-kd protein in all tissues with several structural domains. Three SRC homology domains (SH1-SH3) are located toward the NH_2 terminus. The SH1 domain carries the tyrosine kinase function, whereas SH2 and SH3 domains allow for interaction with other proteins. The C-terminal part of ABL contains a DNA-binding domain, nuclear localization signals, and a binding site for actin (Catherine *et al.,* 1998; Faderl *et al.,* 1999a; Deininger *et al.,* 2000a). Both the SH1 and SH2 protein are required for transformation. DNA-binding and tyrosine kinase activity of nuclear ABL is regulated in a cell cycle dependent manner. ABL is localized in both nucleus and cytoplasm. The normal protein is involved in the regulation of cell cycle, in the cellular response to genotoxic stress and in the transmission of information about the cellular environment through integrin signaling. Overall, ABL serves a complex role as a cellular module that integrates signals from various extracellular and intracellular sources and that influences decisions in regard to cell cycle and apoptosis. Mice with targeted disruptions in the gene have high neonatal mortality rates and increased susceptibility to infections suggesting a role in B-cell development (Raitano *et al.,* 1997). However, *ABL* knockout mice failed to resolve most of the issues (Deininger *et al.,* 2000a). Because of this, the normal function of this gene is not completely understood.

The *BCR* gene occupies a region of about 135 kb on chromosome 22. It is expressed as mRNAs of 4.5 and 6.7, which encodes for the same cytoplasmic 160-kd protein. Like ABL, BCR protein is ubiquitously expressed and has several distinct domains. The first N-terminal exon encodes a serine-threonine kinase, which binds BCR-associated protein-1 (Bap-1), a member of the 14-3-3 family of proteins (Reuther *et al.,* 1994) and possibly BCR itself. This has an important role in signal transduction and cell cycle regulation. A coiled-coil domain at N-terminus allows

dimer formation in vivo (McWhirter *et al.,* 1993). The center of the molecule contains a region with DBL-like and pleckstrin-homology (PH) domains that stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors, which in turn may activate transcription factors such as NF-κB (Deininger *et al.,* 2000a). The C-terminus has GTPase activity for Rac, a small GTPase of the Ras superfamily that regulates actin polymerization and the activity of NADPH oxidase in phagocytic cells (Diekmann *et al.,* 1995; Deininger *et al.,* 2000a). In addition, BCR can be phosphorylated on several tyrosine residues (Wu *et al.,* 1998), especially tyrosine 177, which binds Grb-2 (growth factor receptor-bound protein 2), an important adaptor molecule involved in the activation of the Ras pathway (Ma *et al.,* 1997). Interestingly, ABL has been shown to phosphorylate BCR in COS1 cells, resulting in a reduction of BCR kinase activity (Ma *et al.,* 1997; Deininger *et al.,* 2000a). Although, these data argue for a role of BCR in signal transduction, their true biologic relevance remains to be determined. The fact that the *BCR* knockout mice are viable, fertile and have no obvious defects in hematopoietic cell development and also the fact that an increased oxidative burst in neutrophils is thus far the only recognized defect probably reflect the redundancy of signaling pathways (Raitano *et al.,* 1997; Catherine *et al.,* 1998; Deininger *et al.,* 2000a). If there is a role for BCR in the pathogenesis of Ph-positive leukemias, it is not clearly discernible.

Molecular anatomy of the BCR-ABL translocation

The breakpoints within the *ABL* gene at 9q34 can occur anywhere over a large (greater than 300 kb) area at its 5' end, either upstream of the first alternative exon Ib, downstream of the second alternative exon Ia, or more frequently between the two, and the breakpoints within the *BCR* gene at 22q11 occurs within a 5.8-kb area spanning *BCR* exons 12-16 (Deininger *et al.,* 2000a; Salesse *et al.,* 2002). Depending on the breakpoint in the *BCR* gene, three main types of BCR/ABL genes can be formed (Melo *et al.,* 1996). Regardless of the exact location of the breakpoint, splicing of the primary hybrid transcript yields an mRNA molecule in which *BCR* sequences are fused to *ABL* exon a2. In contrast to *ABL*, breakpoints within *BCR* localize to 1 of 3 so-called bcr. The majority of patients with CML have

breakpoints in introns 1 and 2 of the *ABL* gene and in the major breakpoint cluster region (M-*bcr*) of the *BCR* gene, either between exons 13 and 14 (b2) or 14 and 15 (b3) (Figure 2). Because of alternative splicing, fusion transcripts with either b2a2 or b3a2 junctions can be formed. The final product of this genetic rearrangement is 210 kDa cytoplasmic fusion protein, p210 $^{BCR/ABL}$, which is essential and sufficient for the malignant transformation of CML, and responsible for the phenotypic abnormalities of chronic phase CML (Salesse *et al.,* 2002). Less frequent, the CML is caused by atypical BCR/ABL transcripts. The breakpoints are further upstream in the 54.4-kb region between the *BCR* exons e1 and e2, termed the minor breakpoint cluster region (m-*bcr*). The resultant e1a2 mRNA is translated into a 190 kDa protein (p190 $^{BCR/ABL}$). Recently, a third breakpoint cluster region (μ-*bcr*) was identified downstream of exon 19, giving rise to a 230 kDa fusion protein (p230 $^{BCR/ABL}$), associated with the rare Ph-positive chronic neutrophilic leukemia (Pane *et al.,* 1996; Deininger *et al.,* 2000a).

In contrast to ABL, the BCR-ABL exhibits deregulated, constitutively active tyrosine kinase activity and is found exclusively in the cytoplasm of the cell, complexed with a number of cytoskeletal proteins (Salesse et al., 2002).

These features appear to underlie the ability of BCR/ABL to induce leukemic phenotype. However, the presence of the Ph chromosome in a hematopoietic cell is not in itself sufficient to cause leukemia, because, the fusion transcripts are detectable at low frequency in the blood of many healthy individuals (Biernaux *et al.,* 1996; Bose *et al.,* 1998). It is unclear why Ph-positive leukemia develops in a tiny minority of these persons. It may be that the translocation occurs in cells committed to terminal differentiation that are thus eliminated or that an immune response suppresses or eliminates BCR/ABL expressing cells (Deininger *et al.,* 2000a). Indirect evidence that such a mechanism may be relevant comes from the observation that certain HLA types protect against CML (Posthuma *et al.,* 1999). Another possibility is that, *BCR/ABL* is not the only genetic lesion required to induce chronic-phase CML. Indeed, a skewed pattern of G-6PD isoenzymes has been detected in Ph-negative Epstein-Barr virus transformed B-cell lines derived from patients with CML, suggesting that a Ph-negative pathologic state may precede the emergence of the Ph chromosome (Fialkow *et al.,* 1981).
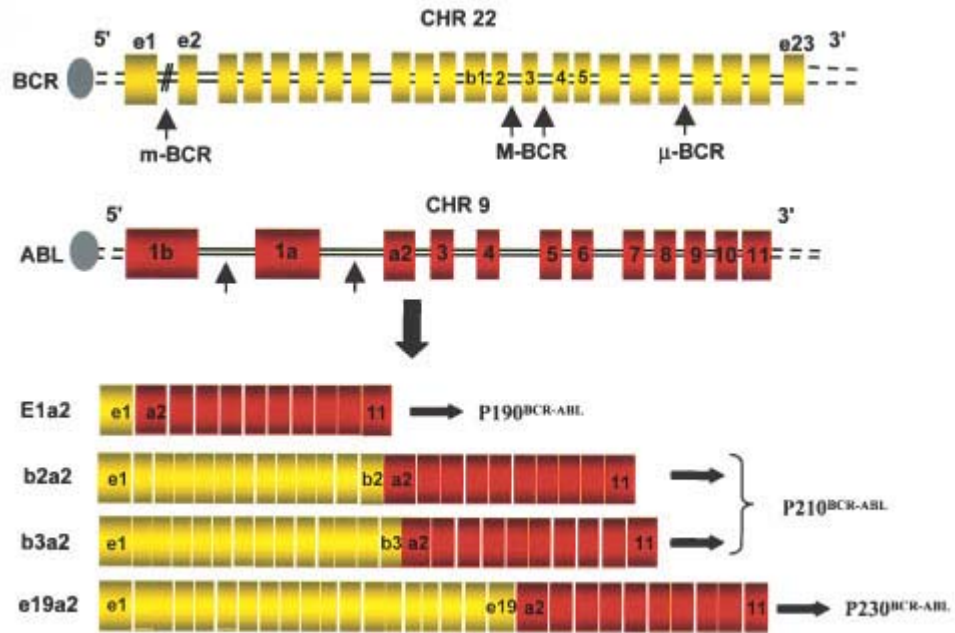
Figure 2: Locations of the breakpoints in the *ABL* and *BCR* genes and structure of chimeric mRNAs derived from the various breaks (Salesse *et al.,* 2002).

Functional domains of BCR/ABL

Several functional domains have been identified in the BCR/ABL protein that may contribute to cellular transformation (Figure 3). In the ABL portion, these domains are the SH1 (tyrosine kinase), SH2 and actin-binding domains; in the BCR portion, they include the coiled-coil oligomerization domain comprised between amino acids 1-63, the tyrosine at position 177 (Grb-2 binding site) and the phosphoserine/threonine rich SH2 binding domain.
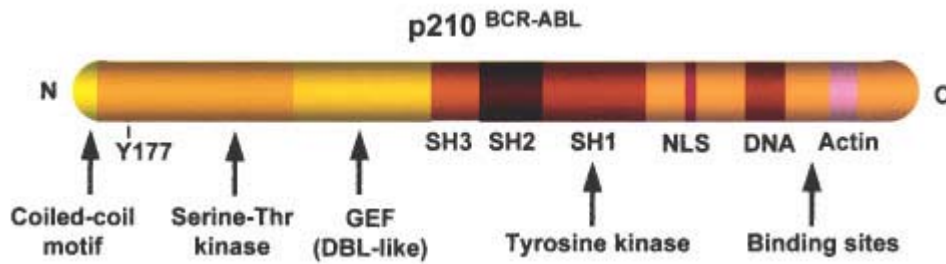
Figure 3: Functional domains of p210 [BCR/ABL] (Salesse *et al.,* 2002). Some of the important domains are illustrated, such as coiled-coil oligomerization domain, the tyrosine 177 (Grb-2 binding site), the phosphoserine/threonine rich SH2 binding domain and the rho-GEF (DBL-like) domain on BCR portion, and the regulatory src-homology regions SH3 and SH2, the SH1 (tyrosine kinase domain), the nuclear localization signal (NLS), and the DNA-and actin-binding domains in the ABL portion.

Physiological properties of BCR/ABL

The physiological properties demonstrated for BCR/ABL are: the induction of neoplastic transformation and cell proliferation, the induction of growth factor independence and inhibition of apoptosis in growth factor dependent hematopoietic cell lines and the inhibition of adhesion of chronic myeloid cells to marrow stroma (Chopra *et al.,* 1999). These processes involve multiple and redundant intracellular pathways.

Ph negative CML

About 9-16% of CML patients are Ph-negative without apparent rearrangement of chromosomes 9 and 22 (Kantarjian *et al.,* 1985; Hild *et al.,* 1990; Chase et al., 2001). In 30-50% of these cases, however, the *BCR/ABL* fusion gene is detectable by molecular analysis (Cortes *et al.,* 1995; Cross, 1997). There is believed to be no prognostic difference between BCR/ABL-positive cases that do or do not have a visible Ph chromosome at diagnosis. The term Ph-negative CML should

therefore be avoided or qualified to indicate whether patients have the BCR/ABL fusion or not. Cases that are Ph-negative and BCR/ABL-negative are heterogeneous but usually have atypical clinical features, respond less well to treatment and show earlier disease progression (Chase *et al.,* 2001).

1.4.3.2 BCR-ABL signaling pathway

The mechanisms implicated in the pathogenesis of CML are altered adhesion to stroma cells and extracellular matrix (Gordon *et al.,* 1987), constitutively active mitogenic signaling (Puil *et al.,* 1994) and inhibition of apoptosis (Bedi *et al.,* 1994; Deininger *et al.,* 2000a).

Increased tyrosine kinase activity of p210$^{BCR/ABL}$ results in the phosphorylation of several cellular substrates and in autophosphorylation of p210$^{BCR/ABL}$, which in turn induces recruitment and binding of a number of adaptor molecules and proteins. Activation of a number of signaling pathways by p210$^{BCR/ABL}$, leads to malignant transformation by interfering with basic cellular processes, such as control of cell proliferation and differentiation (Sawyers, 1993; Afar *et al.,* 1994; Puil *et al.,* 1994; Jiang *et al.,* 2000), adhesion (Gordon *et al.,* 1987; Bhatia *et al.,* 1999) and cell survival (Bedi *et al.,* 1994; McGahon *et al.,* 1994; Cortez *et al.,* 1995*; Cotter,* 1995) (Figure 4).

The structure of p210$^{BCR/ABL}$, allows multiple protein-protein interactions and suggests the involvement of diverse intracellular signaling pathways. In brief, it activates signal transduction pathways such as RAS/MAPK (RAS/mitogen activated protein kinases), PI-3 kinase (phosphotidylinositol 3 kinase), c-CBL (casitas B-lineage lymphoma protein) and CRKL (CRK-oncogene-like protein) pathways, JAK-STAT (Janus kinase-signal transducers and activators of transcription) and the Src pathway. Of these, the RAS that is at the center of the most prominent signaling pathways in CML, Jun-kinase, and PI-3 kinase pathways have been demonstrated to play a major role in transformation and proliferation (Raitano *et al.,* 1995; Sawyers *et al.,* 1995; Skorski *et al.,* 1995, 1997). Inhibition of apoptosis is thought to result from the activation of the PI-3 kinase and RAS pathways, with induction through

AKT (serine/threonine kinase) of c-*myc* and BCL-2 (Raitano *et al.,* 1995; Sawyers *et al.,* 1995; Skorski *et al.,* 1995, 1997; Warmuth *et al.,* 1999b). $p210^{BCR/ABL}$, effects on CRKL, c-CBL, and on proteins associated with the organization of the cytoskeleton and cell membrane, such as paxillin, actin, talin, vinculin and FAK/PYK2 (focal adhesion kinase/ prolin-rich kinase 2) , result in adhesion defects and cytoskeletal abnormalities, characteristics of CML cells (Salgia *et al.,* 1997; Sattler *et al.,* 1998; Sattler *et al.,* 2002).
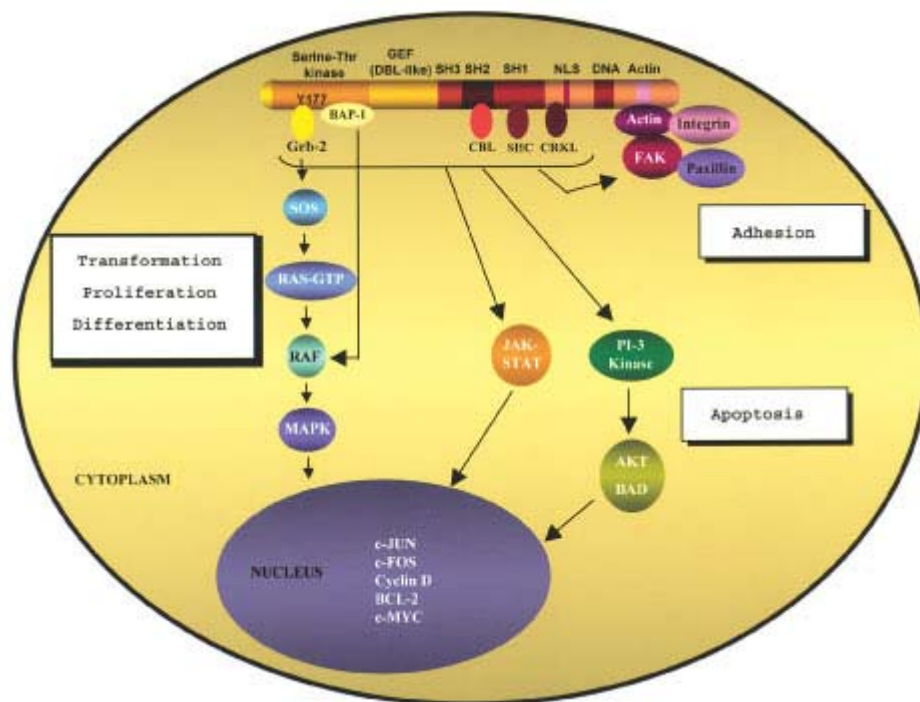
Figure 4: Signaling pathways of p210$^{BCR/ABL}$ (Salesse *et al.,* 2002).

1.4.3.3 Diagnosis and monitoring of CML with *BCR/ABL*

Most commonly all leukemias are diagnosed by a blood test to count the number of red cells, white cells and platelets. Bone marrow aspiration with

measurements of the percentage of blasts and basophils can be done. A biopsy of the bone marrow may also be performed (Faderl *et al.,* 1999b).

The cytogenetic analysis is the gold standard diagnostic test in CML. It is also valuable in demonstrating additional karyotipic abnormalities that occur with disease resistant and transformation (Mitelman, 1993). However, cytogenetic analysis is time-consuming, and only 20-25 cells are examined per sample (Faderl *et al.,* 1999b). In 10% of patients with CML, Ph can not be demonstrated by cytogenetic studies, but molecular analysis will detect *BCR/ABL* rearrangements in up to one half of patients. Genomic polymerase chain reaction (PCR) and Southern blot assay can determine the exact breakpoints of the fusion genes. Reverse transcriptase PCR (RT-PCR) and Northern blot analysis detect *BCR/ABL* transcripts at the RNA level. Western blot analysis or immunoprecipitation demonstrate p210 $^{BCR/ABL}$ protein by using monoclonal antibodies against N-terminal region of BCR and C-terminal region of ABL (Guo *et al.,* 1991).

Monitoring patients who are receiving therapy is commonly done by PCR and fluorescence in situ hybridization (FISH) for *BCR/ABL*. Quantitative RT-PCR is used for follow up of patients after stem cell transplantation (Hochhaus *et al.,* 1998). Despite its sensitivity, RT-PCR may miss Ph-positive cells that are not transcribing the relevant gene product at the time of analysis. FISH allows analysis of metaphase and non-dividing interphase cells and is easily quantifiable. Interphase FISH is performed on peripheral blood. This technique is fast, and it analyzes more cells than is possible with conventional cytogenetic methods. It is therefore, more reliable in assessing cytogenetic responses in CML (Muhlmann *et al.,* 1998). However, it overestimates the degree of cytogenetic response at high Ph-positive percentage values. Because of a false-positive rate of 10%, interphase FISH is not useful once the number of Ph-positive cells decreases to less than 10%. Hypermetaphase FISH allows analysis of up to 500 cells in metaphase per sample in a time-efficient manner without false-positive results, but it requires bone marrow samples other than peripheral blood (Seong *et al.,* 1995). Another FISH technique applicable to blood samples uses double-color probes to detect Ph-positive leukemias and has shown superior sensitivity and specificity (Dewald *et al.,* 1998; Buno *et al.,* 1998).

1.4.3.4 Pathogenesis and transformation of CML

Despite the central influence of BCR/ABL, on the initial development of CML, secondary genetic driving forces mentioned above would presumably important in disease progression (Wetzler *et al.,* 1993; Kantarjian et al., 1997; Faderl *et al.,* 1999c). The disease transformation is often results by refractoriness to treatment, leukocytosis with increases in blood and marrow blasts, basophilia, increases or decreases in platelet counts unrelated to therapy, and clinical manifestations such as unexplained fever, spleenomegaly, extramedullary disease, weight loss, and bone and joint pains.

1.4.4 Treatment of CML

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).

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 drug; and (4) the malignant cells must be destroyed before drug resistance emerges (Wilson *et al.,* 1991).

The natural history of chronic myelogenous leukemia has changed significantly since the first treatment attempts with arsenicals (Fowler's solution) in 1856 by Lissauer. In the past, the median survival of patients with chronic myelogenous leukemia was 3 years; less than 20% of patients were alive 5 years after diagnosis. Survival duration has since doubled to 5 to 7 years; 50% to 60% of patients are alive at 5 years, and more than 30% are alive at 10 years (Kantarjian *et al.,* 1993). Several reasons account for this change: earlier diagnosis; better supportive care; and more effective therapies, such as allogeneic stem-cell transplantation, interferon-α and tyrosine kinase inhibitors (Faderl *et al.,* 1999b; Deininger *et al.,* 1997). Prognostic models based on multivariate analysis allowed stratification of treatment options according to a patient's risk profile, thereby maximizing the chances for the best possible therapeutic outcome (Sokal *et al.,* 1988; Kantarjian *et al.,* 1990; Hasford *et al.,* 1998).

Busulfan, an alkylating agent, controls hematologic variables. In most patients receiving busulfan, disease cannot be controlled safely at the low leukocyte levels needed to induce a complete hematologic response; therefore, partial hematologic response is maintained (Faderl *et al.,* 1999b).

Hydroxyurea, a cell cycle-specific inhibitor of DNA synthesis, became available for the treatment of chronic myelogenous leukemia and it allows rapid but transient hematologic control, is well tolerated, and has few side effects (nausea, vomiting, diarrhea, mucosal ulcers, and skin manifestations) (Faderl *et al.,* 1999b).

Hydroxyurea and busulfan produce complete hematologic remission in 50% to 80% of patients (Kantarjian *et al.,* 1998). Cytogenetic remissions are rare, and both agents do not affect disease progression. Patients will inevitably experience transformation to the blastic phase and die of its complications after a median of 3 to 6 years. Hydroxyurea therapy is superior to busulfan therapy (Faderl *et al.,* 1999b). When these two agents were compared in a randomized study of patients with early chronic-phase chronic myelogenous leukemia, median survival (56 months compared with 44 months) and median duration of chronic-phase disease (47 months compared with 37 months) were significantly longer in the patients receiving hydroxyurea (Hehlmann *et al.,* 1993).

Allogeneic stem-cell transplantation has curative potential in chronic myelogenous leukemia. It produces long-term survival in 50% to 80% of patients and disease-free survival in 30% to 70%. Relapses occur in 15% to 30% of patients, and plateaus are reached at 5 years after transplantation. However, late relapses rarely occur beyond years after transplantation. Applicability of allogeneic stem-cell transplantation is limited by availability of matched siblings and age restrictions. Less than 30% of patients in Europe and North America receive stem-cell transplants from matched sibling donors (Horowitz *et al.,* 1996).

Several factors influence the outcome of stem-cell transplantation. First, younger patients do best with this therapy: Disease-free survival is 60% to 70%, transplant-related mortality is 10%, and probability of relapse is 20% (Faderl *et al.,* 1999b). Older patients do worse mainly because of an increase in treatment-related mortality. Disease-free survival rates are about 30% at 5 years after therapy (Horowitz *et al.,* 1996). Second, disease phase determines outcome. Results are more favorable if patients undergo transplantation during the chronic phase instead of during transformation. Transplantation during advanced phases is characterized by increased rates of leukemia relapse and treatment-related mortality. Third, chemotherapy before transplantation affects disease-free survival. In one study of patients with chronic-phase disease, disease-free survival after transplantation was significantly higher among those who were pretreated with hydroxyurea than among those who were pretreated with busulfan (Goldman *et al.,* 1993). Fourth, the preparative regimen and graft-versus-host disease prophylaxis affect outcome.

Relapse after transplantation occurs in as few as 10% to 20% of patients (those with chronic-phase disease) to as many as 70% to 80% (those with blastic-phase disease and T-cell depletion) (Mrsic *et al.,* 1992; Arcese *et al.,* 1993). Outcome with second transplantations from HLA-identical siblings depends on the time between transplant and relapse. Patients who experienced relapse within 6 months after transplantation had a disease-free survival rate of 7%, a treatment-related mortality rate of 69%, and a probability of relapse of 77%. For patients who experienced relapse more than 6 months after transplantation, the disease-free survival rate was 28%, the treatment-related mortality rate was 30%, and the probability of relapse was 59% (Mrsic *et al.,* 1992).

Interferons are glycoproteins produced by eukaryotic cells in response to antigenic stimuli such as those that occur with viral infections and malignant diseases. They have pleiotropic effects, including antiviral, immunomodulatory, antiproliferative and antiangiogenic activities (Estrov *et al.,* 1993). **Interferon-α (IFN-α)** administration is a promising therapeutic intervention in the chronic phase of CML (Sacchi *et al.,* 1997). It suppresses the leukemic clone and prolongs survival (Faderl *et al.,* 1999a). However, at the advanced stages of the disease IFN-α treatment does not offer a significant therapeutic advantage. Single-arm studies of IFN-α therapy in CML have consistently produced high rates of complete hematologic (46% to 80% of patients) and cytogenetic responses (complete response in 13% to 32% of patients and partial response in 11% to 16%) (Kolb *et al.,* 1995; Kantarjian *et al.,* 1996; Collins *et al.,* 1997; Mahon *et al.,* 1998). IFN-α treatment with chemotherapy confirmed better survival with IFN-α treatment than with either hydroxyurea therapy or busulfan therapy (Faderl *et al.,* 1999b). Five-year survival rates were 57% with IFN-α therapy and 42% with chemotherapy.

Patients with CML who fail IFN-α therapy and who are not candidates for allogeneic stem cell transplantation have limited treatment options and a relatively poor prognosis. **Cytosine arabinoside (ara-C)** have shown single-agent anti-CML activity and have induced hematologic and cytogenetic responses in CML. The combination regimens with ara-C and IFN-α yielded substantially higher rates of complete hematologic and cytogenetic remission in good-risk patients (Thaler *et al.,* 1997). This response translated into significantly longer survival (Guilhot *et al.,* 1997). Combinations of IFN-α with other effective agents (hydroxyurea, busulfan) and intensive chemotherapy were not associated with better results than those seen with IFN-α alone (Kantarjian *et al.,* 1991).

Despite encouraging results with **matched unrelated donor transplantation** (2-year disease-free survival rates, 14% to 43%) (McGlave *et al.,* 1993; Hansen *et al.,* 1998) the procedure carries significant morbidity and mortality rates depending on patient age and degree of matching.

Autologous bone marrow transplantation results in transient cytogenetic responses, but a survival advantage has not been proven (Reiffers *et al.,* 1994). Relapse due to reinfused Ph-positive cells may occur (Deisseroth *et al.,* 1994).

Homoharringtonine is a plant alkaloid derived from the *Cephalotaxus fortuneii* tree. Used in a low-dose continuous infusion, homoharringtonine resulted in complete hematologic response in two thirds of patients and cytogenetic responses in one third (half of whom had major responses). Combinations of homoharringtonine with IFN-α and ara-C are being tested, with promising results (Faderl *et al.,* 1999b).

5-Aza-2'-deoxycytidine (decitabine) is a potent hypomethylating cytidine analogue (Issa *et al.,* 1997). Decitabine produced response rates of 25% in patients with blastic-phase disease and 53% in patients with accelerated-phase disease (Faderl *et al.,* 1999b).

Polyethylene glycol (PEG) interferon is a modified IFN-α molecule that is covalently attached to polyethylene glycol. This interferon has a significantly longer half-life than its parent compound and can be given once weekly instead of daily. In addition to causing fewer side effects, PEG interferon produced a hematologic response in 50% of patients, including 4 of 13 patients who had been resistant to interferon (Faderl *et al.,* 1999b). Preliminary results with PEG interferon are promising, and further investigations are required to validate its role in the treatment of CML.

Antisense oligonucleotides are short DNA sequences modified to bind target RNA sequences within the cell, preventing translation of RNA into functional proteins. *BCR-ABL* antisense oligonucleotides reduce the level of p210$^{BCR/ABL}$ in CML cells and slow the rate of growth and proliferation. Antisense sequences directed against *BCR-ABL* for ex vivo purging in autologous bone marrow transplants are of interest (Ratajczak *et al.,* 1992; De Fabritiis *et al.,* 1998; Maran *et al.,* 1998).

Adoptive immunotherapy: The role of a subset of T lymphocytes in the suppression of leukemic cells is increasingly appreciated. The idea that leukemic cell proliferation is controlled by the immune system is based on several observations. First, the frequency of disease recurrence is increased with T-cell-depleted stem-cell transplantation. Second, donor lymphocyte infusions reestablish cytogenetic remissions in many patients who experience relapse after allogeneic transplantation. Third, a positive correlation has been seen between graft-versus-host disease and reduced risk for relapse after transplantation. Finally, cytogenetic response correlates with interferon therapy-associated autoimmune phenomena (Faderl *et al.,* 1999b).

Research is focusing on identification of specific T-cell clones that can eliminate leukemic progenitors and on proteins that can serve as tumor-specific targets. CML is a good model because p210$^{BCR/ABL}$ is uniquely associated with the Ph translocation (Lim *et al.,* 1997; Moldremm *et al.,* 1997; Choudhury *et al.,* 1997). Identification of leukemia-specific antigens and stimulation of leukemia-specific T-cell responses may allow the use of immunogenicity of leukemic cells in such approaches as immune gene therapy and peptide vaccination (Smit *et al.,* 1997).

Tyrosine kinases are enzymes that transfer phosphate from ATP to tyrosine residues on substrate proteins that in turn regulate cellular processes such as proliferation, differentiation, and survival (Druker *et al.,* 2000). Therefore, **tyrosine kinase inhibitors** are very attractive molecules in CML therapy. Although many molecules along the signaling cascade can be targeted, inhibition of phosphotyrosine kinase activity has been studied most extensively (Levitzki *et al.,* 1995). Natural inhibitors of tyrosine kinases (herbimycin A, genistein, erbstatin, and lavendustin A) have broad specificity for various enzyme substrates. To improve target specificity, synthetic compounds have been modeled after the naturally occurring kinase inhibitors, and more than 20 of them are known (Levitzki *et al.,* 1995). Some of these compounds (AG1112, AG568, and CGP57148B) showed a growth-inhibiting effect on CML cell lines in vitro (Druker *et al.,* 1996; Deininger *et al.,* 1997; Beran *et al.,* 1998; Le Coutre *et al.,* 1999).

Therapeutic agent **STI571** (signal transduction inhibitor number 571) **(Gleevec, imatinib mesylate)** formerly CGP57148B, is a rationally developed, potent and selective inhibitor for ABL tyrosine kinases, including BCR/ABL (Mauro *et al.,* 2001; Goldman *et al.,* 2001). The BCR/ABL tyrosine kinase is a constitutively active kinase, which functions by binding ATP and transferring phosphate from ATP to tyrosine residues on various substrates. This causes the excess proliferation of myeloid cells characteristic of CML. STI571 functions by blocking the binding of ATP to BCR/ABL, inhibiting its activity. As a result, substrates required for BCR/ABL function cannot be phosphorylated and subsequent event are abrogated (Mauro *et al.,* 2001).

The phases I-II clinical trials in CML have demonstrated promising results, especially in the chronic phase of the disease (Etienne *et al.,* 2001;Drummond *et al.,* 2001).

Fifty patients with Philadelphia chromosome-positive CML in early chronic phase received imatinib mesylate, 400 mg orally daily. After a median follow-up of 9 months, 49 patients (98%) achieved a complete hematologic response and 45 patients (90%) achieved a major cytogenetic response, complete in 36 patients (72%) (Kantarjian *et al.,* 2003). Compared with similar patients who received IFN-α with or without hydroxyurea or other IFN-α combination regimens, those receiving imatinib mesylate had higher incidences of complete and major (Ph < 35%) cytogenetic responses at 3 months (34% and 74% versus 1%-4% and 9%-24%, respectively), 6 months (52% and 80% versus 3%-7% and 11%-28%, respectively), and 9 months (60% and 77% versus 5%-11% and 14%-30%, respectively) (Kantarjian *et al.,* 2003a).

An investigation was made whether increasing the dose of imatinib mesylate might overcome drug resistance in patients with Philadelphia chromosome-positive CML whose disease manifests relapse or refractoriness to therapy (Kantarjian *et al.,* 2003b). Fifty-four patients with Ph(+) CML in chronic phase and with hematologic or cytogenetic resistance or relapse on imatinib mesylate therapy at 400 mg orally daily were treated with a higher dose of 400 mg orally twice daily (800 mg daily, 47 patients; or 600 mg daily increased from 300 mg daily, 7 patients). Among 20

patients treated for hematologic resistance or relapse, 13 (65%) achieved a complete (n = 9) or partial (n = 4) hematologic response, but only 1 had a cytogenetic partial response (Ph reduction from 100% to 10%) and 1 had a minor response (Ph reduction from 100% to 50%). Among 34 patients treated for cytogenetic resistance or relapse, 19 (56%) achieved a complete (n = 6) or partial (n = 7) cytogenetic response (Kantarjian *et al.,* 2003b). As a conclusion higher doses of imatinib mesylate may overcome disease-poor response to conventional doses and that this approach deserves further evaluation as frontline therapy for newly diagnosed CML.

237 CML patients were treated with imatinib mesylate at accelerated-phase (Kantarjian *et al.,* 2002). Among the 200 patients with accelerated-phase CML for whom follow-up was 3 months or more, rates of complete and partial hematological response were 80% and 10%. Cytogenetic responses were evident in 90 patients [45%; complete response in 47 patients (24%) and partial response (Ph 1-34%) in 21 patients (11%)]. The estimated 18-month survival rate was 73%. The estimated complete hematological response rate at 18 months was 68%; that for cytogenetic response was 82%. Landmark analysis showed that achieving a cytogenetic response at 3 months or a major cytogenetic response (Ph < 35%) at 6 months was associated with better long-term survival.

Blast crisis is the most advanced stage of CML and is highly refractory to therapy. A total of 260 patients with CML were enrolled in a phase II trial, of whom 229 had a confirmed diagnosis of CML in blast crisis (Sawyers *et al.,* 2002). Imatinib induced hematologic responses in 52% of patients and sustained hematologic responses lasting at least 4 weeks in 31% of patients, including complete hematologic responses in 8% (Sawyers *et al.,* 2002). For patients with a sustained response, the estimated median response duration was 10 months. Imatinib induced major cytogenetic responses in 16% of patients, with 7% of the responses being complete. Median survival time was 6.9 months. These results demonstrated that imatinib has substantial activity and a favorable safety profile when used as a single agent in patients with CML in blast crisis. Additional clinical studies are warranted to explore the efficacy and feasibility of imatinib used in combination with other antileukemic drugs.

As a summary, imatinib mesylate is the new gold standard for treatment of CML. The response rates (both hematologic and cytogenetic) exceeded the rates with other medical therapies (Peggs *et al.,* 2003). However, it remained to be shown whether imatinib therapy was superior to conventional therapy in a direct comparative study, whether the improved response rates translated into improved survival, and whether this treatment could induce molecular remission and possibly be curative in previously untreated patients.

Acquired resistance to imatinib mesylate caused by kinase-domain mutations is common in patients with CML who are treated with the drug (Gorre *et al.,* 2002). Such mutations have been reported only at the time of clinically apparent resistance or relapse (Roche-Lestienne *et al.,* 2002; Shah *et al.,* 2002). A male patient with CML was screened for a mutation of the tyrosine kinase domain of BCR-ABL and was shown to have T315I substitution at diagnosis (Roche-Lestienne *et al.,* 2003). A minor mutated clone present before any treatment seems to have expanded quickly under the selective pressure of imatinib monotherapy.

1.4.4.1 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 *et al.,* 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 disease free survival. Therefore, more precise evaluation of MRD is necessary with regard to therapeutic strategy in monitoring of the disease (Misawa *et al.,* 1995). PCR can be used for detection of MRD in CML (Lee *et al.,* 1988).

1.5 The childhood leukemias

Advances in treatment and prognosis of childhood leukemia are considered a remarkable success of modern medicine. Childhood leukemia, once considered a universally fatal disease, now boasts overall cure rates ranging from 75% to 85% for acute lymphocytic leukemia (ALL) and cure rates approaching 40% to 50% for acute myeloid leukemia (AML) (Colby-Graham *et al.,* 2003). Acute leukemia is the most common form of childhood cancer and is the primary cause of cancer-related mortality in children (Ravindranath, 2003). In the United States, approximately 3250 cases are diagnosed annually in children and adolescents younger than 20 years, of whom 2400 have acute ALL (Ravindranath, 2003).

Cytogenetic and molecular analyses are essential for the classification of childhood hematological malignancies. Nearly all children with leukemia should have an adequate cytogenetic analysis, which in 80-90% is expected to show clonal chromosomal abnormalities (Martinez-Climent, 1997). Moreover, with the availability of appropriate gene probes and sophisticated molecular techniques, genetic rearrangements become detectable in the majority of leukemia patients. Genetic abnormalities often associate with particular clinical-biological characteristics of the disease.

The leukemias of infancy, characterized by an equal distribution of lymphoid and myeloid subtypes, account for 2.5-5% of the ALL and 6-14% of the AML of childhood (Pui *et al*., 1995). Rearrangements of the Mixed-Lineage Leukemia (MLL) gene on chromosome 11q23 are the most common genetic abnormalities in both ALL and AML, occurring in 70-80% and approximately 60% of cases, respectively (Pui *et al*., 1995).

The most significant new development in the past 2 years has been the development of further evidence for fetal origin of childhood leukemias, and additional evidence to support the notion that postnatal events modulating the events of immune-mediated elimination of these leukemic clones play a major role in the eventual development of clinical disease (Ravindranath, 2003). Other epidemiologic developments include (1) increased appreciation of the role of drug-metabolizing

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enzymes, both in determining the predisposition to leukemia and response to therapy; and (2) both clinical observations and gene expression studies seeming to identify a new approach to the evaluation and treatment of children with MLL (11q23) rearrangements (Ravindranath, 2003).

1.5.1 Acute lymphocytic leukemia (ALL)

ALL is primarily a disease of children and young adults. It can be subdivided by morphologic and immunologic criteria. Morphologic subtypes designated as L1, L2, and L3 have been defined in the French-American-British (FAB) classification of acute leukemias (Cheson *et al.*, 1990). 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).

The common cytogenetic abnormalities in Pre-B ALL, T-cell ALL and B-cell ALL are t(9;22), t(4;11), t(1;19); t(8;14), t(7;19), t(1;14); and t(8;14), t(8;22), t(2;8) respectively (Fauci *et al.*, 1998). The *TEL* and *AML1* genes are common targets of chromosomal translocations in hematopoietic malignancies. The *TEL-AML1* fusion gene, created by the t(12;21), is the most common genetic alteration in childhood ALL (28%) and is associated with a favorable outcome (Rubnitz *et al.*, 1999). The exact role of TEL-AML1 oncoprotein in cell transformation remains unclear, but emerging data suggest that the primary effect relates to a compromise of AML1 transcriptional activity, which is required for normal hematopoiesis.

1.5.2 Acute myeloid leukemia (AML)

The term 'acute non-lymphocytic leukemia' (ANLL) is also used for this group of leukemias. AML primarily affect adults between ages of 15 and 39 years and constitutes only 20% of childhood leukemais (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 differentiation. This group of leukemias is of diverse origin. Some arise by transformation of multipotent (trilineage) myeloid stem cells while in others the common granulocyte-monocyte precursor is involved (Figure 1). In the widely used FAB classification, AML is divided into eight categories taking into account both the degree of maturation (M0 to M3) and the predominant line of differentiation of the leukemic stem cells (M4 to M7) (Cotran *et al*., 1989). 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.

Analysis of patient karyotypes reveals that nonrandom, somatically acquired translocations and inversions occur in most acute myeloid leukemias. Among these, fusion oncogenes have been identified that utilize similar signal transduction pathways and transcriptional activation pathways to mediate their leukemogeneic effect (Gilliland *et al*., 2002).   The most common chromosome abnormalities in AML are t(9;22), t(8;21), t(15;17), inversion 16 (inv(16)) or deletion 16q (Cotran *et al*., 1989; Gilliland *et al*., 2002). These translocations may account for up to 30% of all cases of adult and childhood AML and their identification either by cytogenetics or molecular techniques is important for the routine diagnosis and treatment of AML, since they allow the identification of patients whose likelihood of cure is remarkably better (Biondi *et al*., 1996). The t(8;21), resulting in expression of the AML1-ETO oncoprotein, is the most frequent chromosomal abnormality in the myeloid leukemias of both children and adults (Morgan *et al*., 1998). The fusion protein appears to interfere with AML1-mediated transcriptional activation. The CBFbeta subunit is involved in another major rearrangement in AML, the inversion 16, which affects 15 to 18 % of AML cases. This rearrangement joins most of the *CBFB* gene to the carboxyl terminus of the heavy-chain gene of smooth muscle myosin (*MYHII*) Roumier *et al*., 2003).

1.6 DNA Base Excision Repair (BER)

The base excision repair (BER) pathway has a principle role in the repair of mutations caused by reactive oxygen species that are generated during aerobic metabolism (Lindahl, 1993). Although oxidative DNA damage has been implicated in the ethiology of degenerative diseases, aging and cancer (Ames *et al.,* 1991), so far there is no evidence to link inherited deficiencies of BER to these processes.

The excision of some forms of base damage is initiated by the action of a specific class of DNA repair enzymes called DNA glycosylases. These enzymes catalyze the hydrolysis of the N-glycosylic bonds between modified bases and the sugar-phosphate backbone of DNA, leading to removal of the altered bases. Excision of modified bases generates another type of DNA damage, because it results in the formation of sites of base loss called apurinic or apyrimidinic (AP) sites. The repair of AP sites requires further biochemical events to complete BER. The sugar phosphate with the missing base is cut out by the sequential action of AP endonuclease and a phosphodiesterase. The gap of a single nucleotide is then filled by DNA polymerase and DNA ligase (Cheadle *et al.,* 2003).

In *E. coli*, three enzymes help protect cells against the mutagenic effects of guanine oxidation (Michaels *et al*., 1992). MutM DNA glycosylase removes the oxidized base from 8-oxoG:C base pairs in duplex DNA, MutY DNA glycosylase excises adenines misincorporated opposite unrepaired 8-oxoG during replication, and MutT, an 8-oxo-dGTPase, prevents the incorporation of 8-oxo-dGMP into nascent DNA (Cheadle *et al.,* 2003). Homologues of *mutM*, *mutY*, and *mutT* have been identified in human cells and termed *OGG1, MYH* and *MTH1*, respectively (Roldan-Arjona *et al*., 1997; Slupska *et al*., 1996; Sakumi *et al*., 1993).

The *MYH* gene was cloned in 1996 (Slupska *et al.,* 1996). It contains 15 introns and is 7.1 kb long. The 16 exons encode a protein of 535 amino acids that displays 41% identity to the *E. coli* protein (Slupska *et al.,* 1996). The gene maps on the short arm of chromosome 1, between p32.1 and p34.3.

1.7 DNA repair and childhood leukemias

DNA-repair gene mutations have been suspected as being a predisposing factor in the development of leukemia (Horwitz, 1997). One of the first examples of a DNA-repair gene mutation to be causally linked to childhood hematological malignancies and neurofibromatosis type I, is the involvement of a homozygous germ-line mutation in the mismatch repair (MMR) gene *MLH1* (Ricciardone *et al*., 1999; Wang *et al*., 1999). Subsequently, homozygous inactivation of *MSH2*, another MMR gene, was also found to be associated with early onset leukemia (Whiteside *et al*., 2002).

It is well known that heterozygous germline mutations in the MMR pathway genes, *MLH1, MSH2, PMS1, PMS2 and MSH6* lead to hereditary non-polyposis colorectal cancer (HNPCC) (Peltomaki, 2001). Thus, tumorigenesis through an MMR deficiency pathway appears to be associated with two different disease phenotypes, which are dependent on the status of the germ-line mutation: a) HNPCC when the mutation is present on only one allele (heterozygous), and b) hematological malignancies when the mutation(s) is present on both alleles (homozygous or compound heterozygous).

Inherited defects of BER have not been associated with any human genetic disorder, although mutations of the genes *mutM* and *mutY*, which function in *E. coli* BER, leads to increased transversions of G:C to T:A (Nghiem *et al.,* 1998; Michaels *et al.,* 1992; Moriya *et al*., 1993; Thomas *et al*., 1997). Recently, inherited variants of *MYH* was shown to be associated with somatic G:C to T:A mutations in colorectal tumors (Al-Tassan *et al.,* 2002). To determine whether an inherited defect in the 8-oxoG repair pathway was responsible for the pattern of somatic G:C to T:A mutations in family N, Al-Tassan et al. sequenced the ORFs of *OGG1, MYH* and *MTH1* in a blood DNA sample from an affected sibling. Two amino acid variants were identified in MYH (Y165C and G382D), but no likely pathogenic changes were identified in *OGG1,* and *MTH1*. All three affected siblings were found to be compound heterozygotes for Y165C and G382D, and unaffected family members were either heterozygous for one of these variants or normal, suggesting transmission as an autosomal recessive trait. Consistent with this, no somatic mutations in *MYH*

were identified upon comprehensive analysis of the 11 MYH-deficient colorectal tumors (Al-Tassan *et al.,* 2002). In another study, biallelic germline mutations in *MYH* was found to predispose to multiple colorectal adenoma and somatic G:C to T:A muations (Jones *et al*., 2002).

1.8 cDNA microarray technology

The Human Genome Project has spurred the emergence of "genome-wide" approaches to study gene function, regulation and interaction termed functional genomics (Collins *et al.,* 1998). Comprehensive analysis of differential gene expression is becoming a cornerstone for functional genomics. To this end, several new technologies have been developed high-throughput differential gene expression analysis (Schena *et al.,* 1995, DeRisi *et al.,* 1996, Lockhart *et al.,* 1996, Velculescu *et al.,* 1995, Liang *et al.,* 1992, Adams *et al.,* 1991, Hubank *et al.,* 1994). The deposition of cDNAs in an array format to filters or glass is hybridized-based analytical tool at the forefront of this field. The first proof of principle paper showed gene expression analysis for an array with 45 Arabidopsis genes (Schena *et al.,* 1995). This approach has since grown rapidly to high-density microarrays of with tens of thousands of genes. Today, many laboratories around the world have established cDNA microarrays for a range of applications and found it to be very useful for simultaneously profiling mRNA levels for thousands of genes.

The transition of cDNA microarrays from a working prototype to a practical and reliable technology remains the focus of enormous effort from industry and academia. This has required the development and standardization of various hardware, analytical software, statistical methodology, biological resources and biochemical methodologies. Though dream for many laboratories, all of the required functionally essential to reliably use the technology to find changes in gene expression levels between tissues, which provide abundant RNA, has been achieved. The system's performance is currently being evaluated in model systems where observation of known changes and verification of newly observed changes will allow stringent characterization of the reliability with which such profiling can be carried out. The ultimate goal of this effort is to develop the ability to scan the mRNA

expression state of all human genes simultaneously, and to be able to correlate this information with the underlying biochemistry and cellular biology producing the observed biological state.

1.9 Microarray overview

A cDNA microarray experiment can be divided into several stages. First, is the construction of very high density microarrays of specific and distinct DNA hybridization targets, each one representing a single gene, that are spotted in an arrayed format on a glass support. Multiple glass slides, each containing thousands of spots of DNA, can be synthesized and used in subsequent experiments. Then, for each experiment, probes are made which consist of a pool of fluorescently labeled cDNAs. This step begins with the extraction and preparation of mRNAs from two populations of cells. Then, each of the two mRNA is reversed transcribed separately with the incorporation of different fluorescently tagged nucleotides producing two populations of differentially labeled cDNA probes. The two-labeled probes are combined and are then simultaneously hybridized to the cDNA targets on the microarray. A device called reader (scanner) is used to detect the resulting fluorescence of the hybridized probes on the microarray after laser excitation. If a particular gene predominates in one of the two samples, more of the corresponding cDNA will hybridize to the spot representing that gene and color of the fluorescent it is tagged with will predominate. The ratio of the intensity of fluorescence from the two-labeled cDNA on a particular spot is determined using the scanner. The resulting data can then be put into a database and analyzed. Various bioinformatics approaches have been developed to process and visualize the enormous quantity of data generated and also, compare gene expression profiles from multiple experiments. The cDNA microarray schema is summarized in Figure 5.
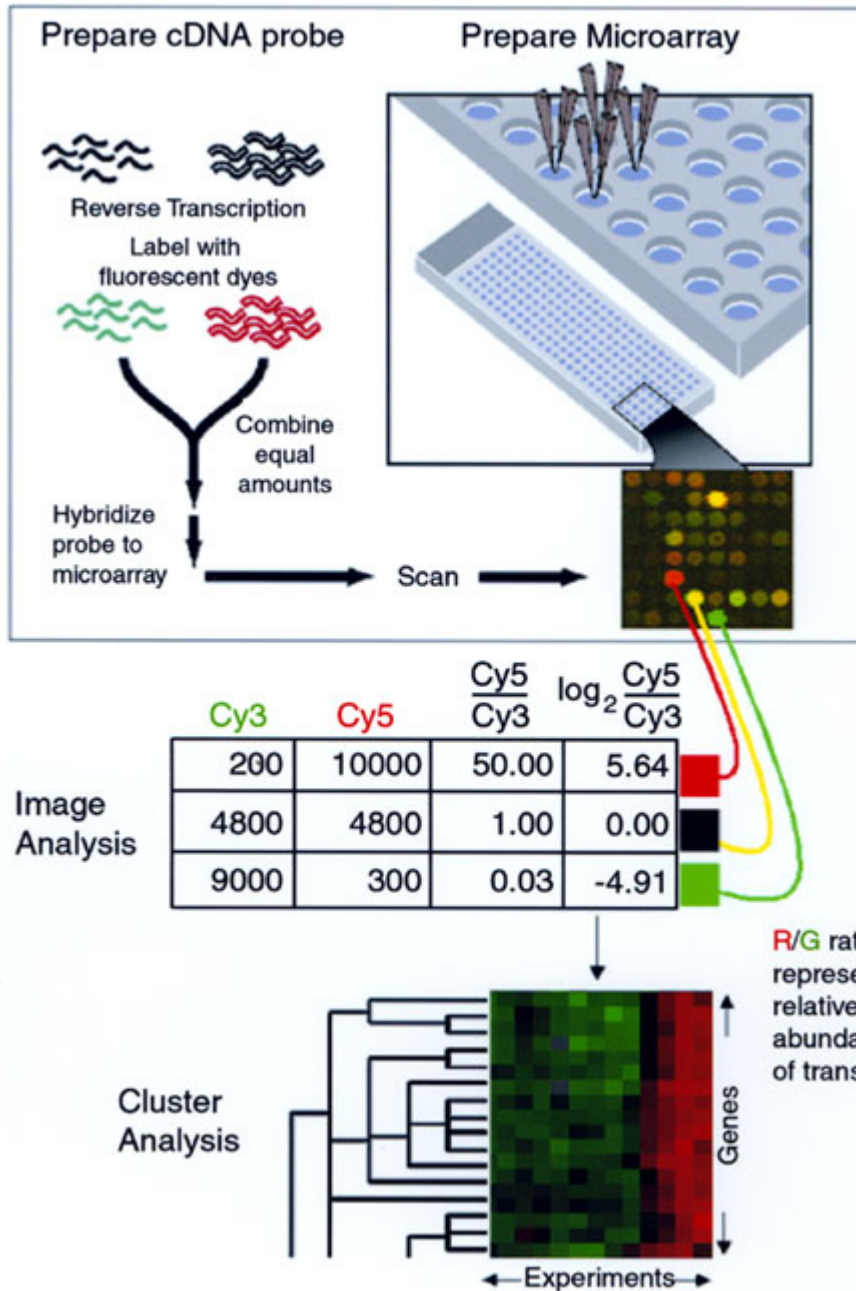
Figure 5: cDNA microaarray schema (Cummings *et al.,* 2000). Capillary printing is used to array DNA fragments onto a glass slide (upper right). RNA is prepared from the two samples to be compared, and labeled cDNA is prepared by reverse transcription, incorporating either Cy3 (green) or Cy5 (red)(upper left). The two labeled cDNA mixtures are mixed and hybridized to the microarray, and the slide is scanned. In the resulting pseudocolor image, the green Cy3 and red Cy5 signals are overlaid--yellow spots indicate equal intensity for the dyes. With the use of image

analysis software, signal intensities are determined for each dye at each element of the array, and the logarithm of the ratio of Cy5 intensity to Cy3 intensity is calculated (center). Positive log(Cy5/Cy3) ratios indicate relative excess of the transcript in the Cy5-labeled sample, and negative log(Cy5/Cy3) ratios indicate relative excess of the transcript in the Cy3-labeled sample. Values near zero indicate equal abundance in the two samples. After several such experiments have been performed, the dataset can be analyzed by cluster analysis (bottom). In this display, red boxes indicate positive log(Cy5/Cy3) values, and green boxes indicate negative log(Cy5/Cy3) values, with intensity representing magnitude of the value. Black boxes indicate log(Cy5/Cy3) values near zero. Hierarchical clustering of genes (vertical axis) and experiments (horizontal axis) has identified a group of coregulated genes (some shown here) and has divided the experiments into distinct classes. (Illustration by J. Boldrick, Stanford University).

Microarray technology is still a new yet immensely powerful tool in molecular biology. The demand for parallel gene expression analysis by cDNA microarray and the dramatic increase in the number of laboratories around the world, which have implemented, established and improved the technology, will lead to its rapid evolution. Improvements are anticipated for every aspect of microarray technology. Differential gene expression experiments have already been successful in a wide spectrum of application to identify single genes and group of genes, which are important in various biological processes. In the future, however, analysis of data across thousands of experiments will enable the elucidation of gene expression fingerprints that can be associated with specific physiological and pathological states. It is becoming clear that, scientists have only scratched the surface of what can be accomplished with the creative application of microarray technology.

1.10 Mutation screening

Detection of unknown mutations can involve DNA sequences analysis of thousands of bases and would require large amounts of money and time. This has lead to the development of many techniques that can be used in research and clinical

laboratories to screen populations for unknown mutations, as well as to detect known mutations.

An optimal mutation detection technique would (1) be fast, (2) be able to screen large stretches of DNA with high sensitivity and specificity, (3) not involve expensive or elaborate instrumentation, (4) not require toxic or dangerous compounds and (5) provide information about the location and nature of the mutation. Unfortunately, no single procedure yet describe possesses all of these attributes.

1.10.1 Restriction enzyme analysis

Restriction endonucleases recognize short DNA sequences and cleave double stranded DNA at specific sites within or adjacent to the recognition sequences. The recognition sequences are generally, but not always, 4 to 6 nucleotides in length and are usually characterized by palindromic sequences. In palindromic sequences the recognition site sequence is the same on each DNA strand when read 5'→3'. Some restriction enzymes cleave at the axis of symmetry yielding "blunt" ends. Others make staggered cleavages yielding "sticky" ends. Restriction enzyme cleavage is accomplished by incubating the enzyme with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of reaction vary depending upon the specific application.

1.10.2 Amplification refractory mutation system (ARMS)

ARMS is also known as PCR amplification of specific alleles (PASA) and allele-specfic PCR (ASPCR) (Wu *et al.,* 1989). It is a generally applicable technique for the detection of known single-base substitutions or microdeletions/insertions. In this PCR-based technique, one of the PCR primers precisely matches one allelic variant of the target sequence, but it is mismatched to the other. When the mismatch occurs at or near the 3' end of the PCR primer, preferential amplification of the

perfectly matched allele is obtained (Sommer *et al.,* 1989). ARMS can generally detect a single copy of a mutant allele in the presence of 40 copies of the normal allele, and may also be used to perform haplotyping of nearby loci in the absence of relatives through double ARMS.

1.11 Aim and strategy

This thesis work had two main objectives. Initially, we focused on gene expression analysis in different risk groups of CML. Furthermore, we investigated the association between *MYH* mutations and childhood leukemia risk.

Specific Aim I:

Although pathophysiologic role of the Philadelphia chromosome translocation in CML has been known for more than 20 years, molecular events that lead to blast crisis - which is seen as the most significant mortality and morbidity factor in CML - is not well understood. At present mutations in genes such as *p53*, *RB1*, *c-MYC*, *p16^{INK4A}*, *RAS*, structural and numerical chromosome anomalies such as trisomy 8, isochromosome i(17q), and an extra Ph chromosome are cited among the genetic abnormalities that appear to contribute to disease progression in CML.

With respect to the development of resistance to treatment in CML, it is known that advanced patients who are at the blastic crisis phase of the disease, and also patients classified as high-risk at the initial diagnosis do not respond to treatment well. The molecular characteristic of three risk groups (low, intermediate and high-risk) of patients is currently unknown. In particular, the relationship between the molecular events characteristics of CML and the biological features of the disease remain to be clarified. Important questions, which need to be addressed, include: 1) What are the genes responsible for the pathogenesis of CML? 2) Are there any gene expression differences between low and high-risk patients at the time of initial diagnosis? 3) Which genes are responsible for high-risk CML and what could be the molecular mechanisms involved? 4) Could response to treatment be predicted at

initial diagnosis? and 5) Is it possible to predict disease outcome by correlating gene expression profiles with survival?

In an attempt to answer these questions, we studied the gene expression profiles in different CML risk groups at the time of initial diagnosis by using cDNA microarrays and quantitative RT-PCR technique to identify candidate CML risk group indicator genes. This profiling may lead to the development of a gene based classification system for CML and may predict disease outcome.

<u>Specific Aim II:</u>

The BER was not linked with any human genetic disorder until recently, when inherited variants of *MYH* was shown to be associated with somatic G:C to T:A mutations in colorectal tumors in a British family. Interestingly, the missense variations Tyr165Cys and Gly382Asp, which significantly reduce the adenine glycosylase activity of MYH protein, were each identified once in a normal control group of 100 British individuals with no history of colorectal adenoma or carcinoma. Since a connection between DNA-repair gene mutations and the path to hematological malignancy is now well established, and individuals who carry heterozygous *MYH* missense mutations Tyr165Cys and Gly382Asp have been documented in a control group, we investigated the association between these two mutations and childhood leukemia risk.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Patient samples

CML patients were referred to Bilkent University, Faculty of Science, Molecular Biology Department (Ankara, Turkey) by collaborating physicians at Ankara University School of Medicine, Department of Hematology and Numune Hospital, Division of Hematology, (Ankara, Turkey). Blood samples were collected in tubes containing EDTA with written informed consent. The cDNAs from other hematological malignancies used in SRT-PCR experiments and DNA samples of AML and ALL were kindly provided by our collaborator Dr. Uğur Özbek, İstanbul University, Institute of Experimental Medicine, Department of Genetics (İstanbul, Turkey). Please see 2.2.1 for detailed information.

2.1.2 Cell lines and tissue culture reagents

Douglas W. Leaman from Borden laboratory, Center for Drug Discovery and Development, Taussig Cancer Center, Cleveland Clinic Foundation, Cleveland, USA, kindly supplied K562 cell line (myeloid cell line originally derived from a CML patient at blastic crisis). RPMI 1640, fetal calf serum was obtained from BIOCHROM AG Seromed (Berlin, Germany). Magnesium-free phosphate buffered saline (PBS) and Penicillin / Streptomycin mixture was from Biological Industries (Haemel, Israel). Tissue culture flasks, petri dishes, 15 ml polycarbonate centrifuge tubes with lids and cryotubes were purchased from Costar Corp. (Cambridge, England).

2.1.3 Oligonucleotides

The oligonucleotides used in semi quantitative reverse transcription polymerase chain reaction (SRT-PCR), real-time RT-PCR and mutation screening were synthesized by İONTEK (Bursa, Turkey). The consensus primers (AEK M13 and pSPORT) used in the array experiments for target cDNA preparations were from Life Technologies (MD, USA). Oligo dT(15)-T7, oligo dT18 and TS primers used in mRNA amplification were supplied from Williams laboratory, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, USA. Random primers were from GibcoBRL Life Technology Inc. or Invitrogen Life Technologies (MD, U.S.A). Sequence of the primers used throughout this study is given in Table 1.

Table 1: List of primers and PCR conditions

Primer	Sequence (5'→3')	Product size (bp)	Tm value (°C)	Used in	Target Gene/vector
AEK M13 / F AEK M13 / R	GTTGTAAAACGACGGCCAGTG CACACAGGAAACAGCTATG	Depends on size of cDNA insert	55	target cDNA preparation for arrays	*RG* *clones*
pSPORT / F pSPORT / R	CCAGTCACGACGTTGTAAAACGAC GTGTGGAATTGTGAGCGGATAACAA	Depends on size of cDNA insert	65	target cDNA preparation for arrays	*RG clones*
CCNH / F CCNH / R	GGTTCTTCCGAATGATCCAG CTGTTCAAGTGCCTTCTCCT	310	55	SRT-PCR	*CCNH*
CXCL3/ F CXCL3/ R	ACCAACTGACAGGAGAGAAG GGTGGCTGACACATTATGGT	344	55	SRT-PCR	*CXCL3*
IFITM1 / F IFITM1 / R	TG CACAAGGAGGAACATGAG CTGTTACAG AGCCGAATACC	330	55	SRT-PCR Real-time RT-PCR	*IFITM1*
PRKAR2B/ F PRKAR2B/ R	TCACAAGGCGTGCCTCAGTAT CAGTGATTGTAGCTGCTCTGG	387	55	SRT-PCR	*PRKAR2 B*
PTGS1 / F PTGS1 / R	CTCTGGTTCTTGCTGTTCCTG CATGTGCTGAGTTGTAGGTGG	382	55	SRT-PCR	*PTGS1*

RAB1A / F RAB1A / R	GACTACACAACAGCGAAGGA ACCTGACTGCTTGACTGGAG	201	55	SRT-PCR	*RAB1A*
UCP2 / F UCP2 / R	CTGCATCGCA G ATCTCATCAC ATAGGTCACCAGCTCAGCACA	520	55	SRT-PCR	*UCP2*
GAPDH / F GAPDH / R	GGCTGAGAACGGGAAGCTTGTCAT CAGCCTTCTCCATGGTGGTGAAGA	250 from gDNA 151 from cDNA	55	SRT-PCR Real-time RT-PCR	*GAPDH*
TS (template switch) oligo primer	AAGCAGTGGTAACAACGCAGAGTACG CGGG	-	-	mRNA amplification	-
Oligo dT(15)-T7	AAACGACGGCCAGTGAATTGTAATAC GACTCACTATAGGCGC T(15)	-	-	mRNA amplification	-
Y13 / F Y13 / R	AGGGCAGTGGCATGAGTAAC GGCTATTCCGCTGCTCACTT	242	57	PCR for restriction enzyme analysis	*MYH*
165N/ normal 165M/ mutant 165C/ common	CGCCGGCCACGAGAATGGT CGCCGGCCACGAGAATTGC AGTGCTTCCCTGGAGGTGAGA	147	62	ARMS	*MYH*

2.1.4 Enzymes

Ribonuclease H (RNase H) was from GibcoBRL Life Technology Inc. (Gaithersburgs, MD, U.S.A). Anti-RNase was from Ambion Inc (TX, USA). *Taq* DNA polymerases were supplied from Perkin Elmer (USA) and MBI Fermentas (Germany). *Bgl* II was supplied from MBI Fermentas (Germany).

2.1.5 Thermal cyclers

The GeneAmp System 9600 (Perkin-Elmer, USA) or DNA Engine Tetrad, PTC-225 (MJ Research Inc., MA, USA) or Mastercycler Eppendorf Scientific, Inc. (NY, USA) thermal cyclers were used in this study. iCycler instrument was from BioRad Laboratories (CA, U.S.A).

2.1.6 cDNA clones

The microarrays utilized in this study contain 10,750 unique cDNAs from IMAGE consortium clones (Research Genetics, Huntsville, AL) (Ross *et al.,* 2000; Frevel *et al*., 2003). These are provided as *E. coli* cultures in 96-well plates. The constructs pGIBS-PHE, pGIBS-THR, pGIBS-TRP and pGIBS-DAP used in the preparation of positive control probe (spike controls) from *Bacillus subtilis* were from American Type Culture Collection (ATCC, VA, USA).

2.1.7 Chemicals, reagents and kits

All laboratory chemicals were analytical grade from Sigma Biosciences Chemical Company Ltd. (St. Louis, U.S.A), Farmitalia Carlo Erba (Milano, Italy), Merck (Schucdarf, Germany), MBI Fermantas (Germany) and Delta Kim Sanayi ve Ticaret A.S (Turkey). TRIzol Reagent and Superscript II RNase H⁻ Reverse Transcriptase were from GibcoBRL Life Technology Inc. (Gaithersburgs, MD, U.S.A). RNA/DNA Stabilization Reagent, Phenol:Chloroform:Isoamyl alcohol were

from Boehringer Mannheim. T7 Megascript kit, DNA-free kit, Linear acrylamide, and Lithium chloride precipitation solution were from Ambion Inc (TX, USA). Phase Lock Gel (PLG) was from Eppendorf Scientific, Inc. (NY, USA). Micro Bio-Spin 6 Chromatography Columns, Tris were from Bio-Rad (CA, USA). GFX PCR DNA - Gel Band Purification Kit (GFX columns) was from Amersham Pharmacia Biotech. Inc. (NJ, USA). QIAquick PCR purification kit was from Qiagen (CA, USA). Advantage cDNA Polymerase Mix was from Clontech laboratories (CA, USA). Deoxyribonucleotide triphosphates (dNTPs) and RevertAid First Strand cDNA synthesis kit were from MBI Fermentas (Germany). LightCycler-DNA Master SYBR Green I was from Roche, Molecular Biochemicals (Germany).

2.1.8 Standard solutions and buffers

DEPC-treated water (DEPC ddH$_2$0): 0.1% Diethylpyrocarbonate (DEPC) (v/v) in double-distilled water was stirred in loosely plugged bottle. Then autoclaved and stored at room temperature.

5X Formaldehyde gel running buffer: *Per liter:* 20 ml of 2M Sodium Acetate, 20.6 gr MOPS, 780 ml of DEPC treated distilled water. pH was adjusted to 7.0 with 5M NaOH. Then 10 ml of 0.5 M EDTA pH 8.0 and volume was completed to 1 liter with DEPC-treated water. Filtered through 0.2 μm filter and stored at room temperature in the dark.

RNA loading buffer: 50% formamide, 20% Formaldehyde, 15% 5X running buffer, 15% glycerol-dye. Stored at -20 °C or 50% glycerol, 1mM EDTA pH 8.0, 0.25%

bromophenol blue, 0.25% xylene cyanol in DEPC ddH$_2$0.

1XTAE (Tris-acetic acid-EDTA):	40mM Tris-acetate, 2mM EDTA, pH 8.0
1XTBE(Tris-boric acid-EDTA): 2mM EDTA, pH 8.3	89mM Tris-base, 89mM boric acid,
20XSSC:	3M NaCl, 0.3 M trisodium citrate, pH 7.0
TE Buffer:	10mM Tris HCl, 1mM EDTA, pH 8.0
Ethidium bromide:	10 mg/ml in water (stock solution), 30 ng/ml (working solution)
5X Gel loading buffer:	0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol, 1mM EDTA
10X Phosphate-buffered saline (PBS):	*Per liter:* 80 g NaCl, 2 g KCl, 14.4 g Na$_2$HPO$_4$, 2.4 g KH$_2$PO$_4$, pH 7.4
Luria-Bertani medium (LB):	*Per liter:* 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl.
Ampicillin:	100 mg/ml solution in double-distilled water, sterilized by filtration and stored at -20°C (stock solution). 200 μg/ml (working solution)
Penicillin/streptomycin mixture:	100 U/ml Penicilin, 0.1 mg/ml streptomycin

Glass cleaning solution: 400 ml ddH$_2$0, 100 g NaOH and 600 ml 95% ethanol. First, NaOH pellets were dissolved in water, and then ethanol is added and stirred until the solution is clear. If the solution does not clear, ddH$_2$0 is added until it does.

Poly-L-lysine solution: 60 ml poly-L-lysine (0.1%w/v solution from Sigma), 60 ml 1XPBS and 480 ml ddH$_2$0.

Blocking solution: 170 mM succinic anhydride, 70 mM sodium borate in 1-methyl-2-pyrrolidinone. (11.08 g succinic anhydride was dissolved in 600 ml 1-methyl-2-pyrrolidinone with stirring bar, and finally 46.1 ml 1M Borate buffer was added to the solution just before dipping the slides).

2.1.9 Nucleic acids

DNA molecular weight standards ∅X174 DNA/HinfI Marker, 10 and pUC Mix Marker, 8 were supplied by MBI Fermentas (Germany).

2.1.10 Fluorescent dyes

FluoroLink Cy3/Cy5 monofunctional NHS-ester dyes and FluoroLink Cy3/Cy5-dUTP (Cy3/Cy5-AP3-dUTP) dyes were supplied from Amersham Pharmacia Biotech. Inc. (NJ, USA) and stored at –20°C in the dark.

2.1.11 Electrophoresis and photography

Electrophoresis grade agarose was supplied from Sigma Biosciences Chemical Company Ltd. (St. Louis, MO, U.S.A). Horizontal electrophoresis apparatuses were from Stratagene (Heidelberg, Germany) and E-C Apparatus Corporation (Florida, U.S.A). The power supply Power-PAC300 was from Bio Rad Laboratories (CA, U.S.A). The Molecular Analyst software used in agarose gel profile visualizing was from BioRad Laboratories (CA, U.S.A).

2.2 Methods

2.2.1 Sample collection and clinical scoring

Blood was obtained from CML patients and four apparently healthy volunteers with written informed consent and left in EDTA tubes, in vertical position, overnight at $4^{o}C$ until the serum, leukocytes and erythrocytes were separated. Buffy coats were aspirated, washed with 1xPBS and stored in RNA/DNA Stabilization Reagent (Boehringer Mannheim) at $-80^{o}C$ until RNA isolation.

Sixty-seven chronic myeloid leukemia (CML) patients were classified according to new prognostic score (NPS) (Hasford *et al.,* 1998). At the time of initial diagnosis CML patients can be divided into three groups of low-risk, intermediate-risk, and high-risk, based on certain clinical parameters such as age, spleen size, blast, platelet, eosinophil, and basophil counts. The scoring was done according to the formula below:

NPS = **(**0.6666 x age [0 when age <50 years; 1, otherwise] + 0.0420 x spleen size [cm below costal margin] + 0.0584 x blasts [%] + 0. 0413 x eosinophils [%] + 0.2039 basophils [0 when basophils < 3%; 1, otherwise] + 1.0959 x platelet count [0 when platelets < 1500 x 10^{9}/L; 1, otherwise]) x 1000.

is CML score calculator site, which can also be used for NPS calculation automatically. NPS for low-risk, intermediate-risk, and high-risk CML are ≤ 780 with median survival time of 98 months, > 780 and ≤ 1480 with median survival time of 65 months, and > 1480 with median survival time of 42 months, respectively. The patient information and informed consent forms that were filled at the time of blood collection are given in Appendix (a) and (b), respectively.

185 cases of childhood leukemias subdivided into two groups, AML; n=45 and ALL; n=140 were diagnosed at Istanbul University between 1998 and 2002. The French-American-British Cooperative Study Group criteria were used for histological subgroup classification (Cheson *et al*., 1990). Randomly selected Bilkent University students, with no history of hematological malignancies or any other type of cancer (n=124) were used as healthy controls.

2.2.2 RNA isolation, DNaseI treatment and control RNA

For gene expression analysis in CML it is important to include all types of leukocytes since CML involves cells from multiple heamatopoietic lineages (Faderl *et al*., 1999a). Therefore, RNA was isolated from the buffy coat. Consequently, our results represent gene expression from whole blood leukocytes. Total RNA was extracted by TRIzol Reagent (GibcoBRL) in 14 ml polypropylene round-bottom tube. For 5-10 X 10^6 cells, 1 ml TRIzol was used. The sample was mixed well by repetitive pipetting and incubated for 5 min at 15 to 30^oC to permit the complete dissociation of nucleoprotein complexes. 200 μl chloroform was added for per 1 ml of TRIzol reagent. The tube was shaken vigorously by hand for 15 sec, incubated at 15 to 30^oC for 2-3 min and centrifuged at 9,200 rpm for 15 min at 4^oC. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase. RNA remained exclusively in aqueous phase. Because of this, it was transferred to a fresh tube. The RNA was precipitated by mixing with 500 μl isopropyl alcohol per 1 ml of TRIzol used initially. The sample was incubated for 10 min at 15 to 30^oC and centrifuged at 9,200 rpm for 10 min at 4^oC. The supernatant was removed and the RNA pellet was

washed once with 1 ml 75% ethanol. The sample was mixed by vortexing and centrifuged at 7,250 rpm for 10 min at 4°C. The pellet was air-dried for 5-10 min, dissolved in 20-30 µl RNase-free water by passing the solution a few times through a pipette tip, incubated at 55-60°C for 10 min, transferred to 1.5 ml tube and stored at – 80°C.

The total RNA isolated was treated with DNaseI (DNA-free kit, Ambion) following the manufacturer's directions. Briefly, 1/10 volume of 10X DNase I buffer and 1µl DNase I (2 U/µl) was added to the RNA mix and incubated at 37°C for 45 min. The DNase inactivation reagent was resuspended by flicking or vortexing the tube. 1/10 volume of slurry solution was added to the sample, the tube was flicked to disperse the reagent in the RNA preparation and incubated at room temperature for 2 min. The tube was centrifuged at 10,000 rpm for 1 min to pellet the DNase inactivation reagent and the supernatant was transferred to a fresh tube. The quality/the concentration of RNA was measured by spectrophotometer (OD260/280) and all RNAs were run on denaturing agarose gel.

Lithium chloride (LiCl) precipitation (Ambion) was performed for some CML samples while doing optimization experiments following the manufacturer's directions. This is an effective way to remove carbohydrates, some small RNAs and also genomic DNA. One-half final volume of LiCl precipitation solution was added to RNA sample, mixed well and stored at –20°C for at least 30 min or overnight. After the centrifugation at 13,000 rpm for 15 min at 4°C, the supernatant was discarded. Residual salts were removed by washing the pellet with EtOH. 300 µl 75% EtOH was added per 1.5 ml tube and the tube was flicked for about 1 min. The RNA was recovered by centrifugation at 7,500 rpm for 10 min at 4°C, air-dried and resuspended in 10-20 µl DEPC-H$_2$O.

Universal Human Reference RNA (Stratagene) was used as a control RNA in microarray experiments, allowing comparison of relative gene expression across samples. This is a high-quality pool of total RNA for human microarray gene expression profiling and is a standard for accurate and consistent data comparison between different experiments. The reference RNA cell lines providing consistent

and reproducible RNA populations include testis, brain, liver, skin, breast, cervix, T and B-cells, macrophages, and lipocytes.

2.2.3 *BCR-ABL* analysis

BCR-ABL fusion gene analysis were done by fluorescence in situ hybridization (FISH) (Vysis, Inc. USA) and RT-PCR (Roche, Molecular Biochemicals) to confirm t(9;22) (q34;q11) at Ankara University, School of Medicine, Department of Hematology and at İstanbul University, Institute of Experimental Medicine, Department of Genetics. All patients were positive for p210$^{BCR-ABL}$.

2.2.4 Treatment of K562 cells with Interferon α-2b and extraction of total RNA

K562 cells were cultured in RPMI-1640 with 10% fetal calf serum, 1% Penicillin / Streptomycin mixture and 5% CO_2 at 37°C. The cells were plated at 2 x 10^6 cells/ml in 50 ml of medium overnight before being treated with 1,000 U/ml interferon α-2b (IFNα-2b) (Roche, specific activity, 2.7 x 10^8U/mg) for 16 hours. One plate was left untreated and used as a control. After treatment, the cells were collected by centrifugation at 3,000 rpm for 3 min and the total RNA was isolated by TRIzol reagent as mentioned in detailed above.

This optimization experiment was done basically to check the arrays before using them for patient samples and also to find out the amount of amplified RNA that will be used in microarray experiments.

2.2.5 Agarose gel electrophoresis of nucleic acids

Horizontal agarose gels of DNA samples

DNA fragments were fractionated by horizontal electrophoresis by using standard buffers and solutions. DNA fragments less than 1 kb were generally separated on 1.0 % agarose gel, those greater than 1 kb (up to 11 kb) were separated on 0.8 % agarose gels.

Agarose were completely dissolved in 1x TAE (or 1xTBE) electrophoresis buffer to required percentage in microwave and ethidium bromide was added to final concentration of 30 ng-1 µg/ml. The samples were loaded onto agarose gel with 1/5 volume of loading buffer. The gel was run in 1x TAE (or 1xTBE) at different voltage and time depending on the size of the fragments at room temperature.

Gel electrophoresis of total RNA

RNA was fractionated through 1% (w/v) agarose gels containing formaldehyde, which disrupts hydrogen bonds. 0.75 g agarose was melted in 46.6 ml DEPC H_2O, allowed to cool to 60°C. 15 ml of 5X formaldehyde gel running buffer and 13.5 ml of 37% (12.3M) formaldehyde were added. The gel was immediately poured in a laminar hood. 5µl of RNA sample was mixed with 15 µl of RNA loading buffer and heated at 70 °C for 5 minutes. Samples were quenched on ice and loaded onto gel. Electrophoresis was performed at a constant voltage (85 V) for 4 hr at 4 °C in 1X formaldehyde gel running buffer. Following electrophoresis, gel was soaked for 5 min in 5 volumes water to remove formaldehyde. This step was repeated for 3 times. The gel was stained in 30ng/ml ethidium bromide solution for 5 min, and destained overnight in double-distilled water.

Nucleic acids were visualized under ultraviolet light (long wave, 340 nm) and DNA size markers (MBI Fermentas) were used to estimate the fragment sizes.

2.2.6 Quantification and qualification of nucleic acids

Concentration and purity of the double stranded nucleic acids, oligonucleotides and total RNAs were determined by using the Beckman Instruments

Du Series 600 Spectrophotometer software programs (ds DNA ,Oligo DNA Short and RNA methods) on the Beckman Spectrophotometer Du640 (Beckman Instruments Inc. CA. U.S.A).

2.2.7 Construction of arrays

The steps to prepare cDNA arrays are as follows which is described in details in the laboratory manual by Mousses S. et al, Cancer Genetics Branch, National Human Genome Research Institutes, NIH, USA.

2.2.7.1 Preparation of target DNA

The choice of which genes, and how many will be printed on a glass microarray depends on the availability of clones and sequences, the capacity of the slide, but ultimately on the purpose of the experiment. For this project, 10,750 cDNAs from IMAGE consortium clones (Research Genetics) were prepared. The sequence-verified for these were obtained from 40K human clone set. The microarray contained all available genes from BRG Williams laboratory (CCF, LRI, Cleveland, USA), including AU-rich genes, interferon stimulated genes, house keeping genes, ESTs and four positive control sequences of bacterial origin.

2.2.7.1.1 Isolation of clones from bacteria

The bacteria (2 μl) that has the gene of interest from Research Genetics Plates (stored at –80°C and thawed on ice before growing) were grown in ampicilin (200μg/ml) containing 1200 μl LB at 37°C, 300 rpm overnight, in fresh 96 well plates (round bottom). The glycerol stocks were prepared by transferring 100 μl grown culture into 100 μl 60% glycerol in 96 well plates (Evergreen Scientific, USA) and stored at –80°C. In order to prepare PCR templates, 200 μl grown culture was transferred into PCR microplate 96-L-C (AxyGen Inc., USA) the lid was put on

and the plate was centrifuged at 3,200 rpm for 15 min. The supernatant was discarded carefully and the pellets were stored at –20°C until further use.

Plasmid DNA preparation (minipreps) from cultures can be done by using QIAprep 96 Turbo Miniprep Kit from Qiagen or Edge BioSystems 96 well Alkaline Lysis Miniprep Kit, in order to improve the success rate, yield and efficiency of PCR. However, there is another simple method for template preparation, which is time efficient, cost effective, and has a success rate near that of purified plasmid. 150 μl TE buffer was added to pellet of bugs, boiled at 98°C for 8 min and cooled at 4°C for 10 min. After pelleting the debris by centrifugation at 3,200 rpm, for 15 min, the supernatant was transferred to new 96 well PCR plates and used as a template in PCR.

2.2.7.1.2 PCR amplification and purification of clones

5 μl template DNA was used in 100 μl PCR reaction containing 1X PCR buffer (10mM Tris-HCl (pH 8.3), 50 mM KCl), 1.5 mM MgCl$_2$, 200 μM dNTPs, 0.5 μM from each primer (AEK M13 and/or pSPORT primers, Table 1) and 1 U *Taq* DNA polymerase (PE Biosystems, CA, USA). Amplification was done using DNA Engine Tetrad, PTC-225 (MJ Research Inc., MA, USA) or Mastercycler Eppendorf Scientific, Inc. (NY, USA) thermal cyclers under the following PCR conditions:

For AEK M13 primers; initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing), 72°C for 1 min (extension) and a final extension at 72°C for 5 min. For pSPORT primers; initial denaturation at 95°C for 1 min, 38 cycles of 94°C for 30 sec (denaturation), 65°C for 45 sec (annealing), 72°C for 3 min (extension) and a final extension at 72°C for 3 min. After the PCR cycles were completed, the tubes were held at 4°C for at least 5 min or until removal. 5 μl from PCR products were run on 1.5% agarose gel in 1x TAE and visualized under UV transilluminator.

PCR products were then purified with size exclusion filter plates (Milipore MANU 030 PCR). Simply, the remaining products were transferred to purification plates and vacuumed approximately 5 min, sucking two times from bottom with paper towel until the filters were shiny. 100 μl sterile ddH$_2$O was added to all and vacuumed again in the same way. Next, 65 μl sterile ddH$_2$O was added and the plates were put onto shaker (Orbital Shaker, Bellco, NJ, USA) for 10 min at speed 5. After, the samples were transferred to 96 well plate, quality controlled by agarose gel electrophoresis again and stored at –20°C until further use for preparation of spotting DNA plate.

2.2.7.1.3 Preparation of spotting cDNAs

5 μl filtered, 3X SSC was added to 384 well polypropylene, V bottom, microplate (Whatman Inc., NJ, USA). Then, 5 μl from each cDNA product was transferred to microplate and mixed. The final concentration of SSC must be 1.5X for the spotting plates. Finally, the plates were wrapped with moist paper towel to prevent evaporation, put in plastic bags, sealed and stored at –20°C.

2.2.7.2 Printing of DNA target microarrays on glass slides

2.2.7.2.1 Treatment of glass slides (poly-L-lysine coating)

Plain Gold Seal Microscope Slides (Gold Seal Products, NH, USA) were used because, these slides have consistently low intrinsic fluorescence. Treatment of slides with poly-L-lysine prior to printing allows the target DNA to adhere to the surface (poly-L-lysine provides a positively charged surface for the negatively charged cDNA to bind) and minimize loss during hybridization. Powder free gloves must be worn all times and detergents or other compounds that may cause background fluorescence must be kept away from slides.

Slides were placed into 50-slide rack (stainless steel/glass) and fresh cleaning solution was prepared (400 ml ddH$_2$0, 100 g NaOH and 600 ml 95% ethanol). The

rack was submerged in the cleaning solution in a glass tank/boat by shaking for 2 hours. The slides were then removed and rinsed with ddH$_2$0 5 times, 5 min each. The clean slides were placed into 25-30-slide rack (plastic or glass) so that they can be centrifuged after coating. The poly-L-lysine solution was prepared freshly and the rack was submerged into the solution by shaking for 1 hour. The rack was rinsed once in ddH$_2$0 for 1 min and centrifuged for 5 min in a low speed (1000 rpm) swinging holder centrifuge to remove free liquid. After this, the slides were transferred to a clean plastic slide box immediately. The lid of the box must be kept open in a clean drawer overnight to avoid moist on the slides. Usually, the slides were allowed to age for 1-2 weeks or at least 4-5 days before printing. Aged slides will be very hydrophobic (water drops leave no trail when they move across surface).

2.2.7.2.2 Spotting with arrayer

The cDNAs were printed with SDDC-2 Microarrayer (Virtek Vision Inc.) and the software used was ArrayMaker version 1. Every gene probe was spotted in duplicate so that each array gives at least two data points for each gene. This is helpful when determining the amount of variation one can expect in a single hybridization. The SDDC-2 Microarrayer is the most precise arrayer on the market. It is truly modular and reconfigurable. The basic system has a robot carrying a dispenser head with up to 48 stealth pins. The minimum spot size is 75 μm, minimum spotting center-to-center distance is 120 μm and maximum number of spots per slide is approximately 80,000. The average production of gene arrays with SDDC-2 is 1 sec/spot/pin. The main sub-systems of the arrayer are as follows:

3-Axis Robot Gantry Sub-system: This unit is one of the key factors that give the SDDC series the leading edge above competing systems. The preloaded ball screws provide negative compliance resulting in zero deviation under the effective loads during the robot operations. The zero backlash transmission in the X, Y, and Z directions is combined with most sophisticated digital AC servo drives and special encoders. Their integration is based on extensive analysis of structural deflections, temperature effects, acceleration forces, and optimum torque requirements. Weight minimization of end-effector and each subsequent axis has resulted in nearly zero

deflection structure. **Dispenser and Pipettor Sub-system**: The SDDC series can be used with different types of dispensing and liquid transfer systems for the different needs (micro-arraying, pipetting). **Cleaning and Vacuum Sub-system**: A vacuum based cleaning sub-system for use with any dispenser is provided. The vacuum chamber is designed with matching holes for the offered array. Its dispensing head (up to 48 pins), such that an optimum gap between pins and holes results in effective suction. Before and after vacuuming, the pins are dipped in water container. The number of times this cycle is to be repeated is programmable.

Slide and Source Plate Holder Platen Standard: Size platen can accommodate one microwell source plate (96- and 384-well) and up to 75 of 25 mm x 75 mm slides. As well, 25 mm x 25 mm, 50 mm x 75 mm, or 75 mm x 75 mm slides can be used. Large platen can accommodate up to 126 slides. The modular version of the standard platen can accommodate up to 8 adapter plates instead of the slides. An adapter plate can hold a microwell plate, membrane or 5 slides. The modular version of the large platen can accommodate up to 15 adapter plates.

Automatic Microwell Plate Stacker: Internal plate stacker with a capacity of 18 plates is used to achieve automatic loading of microwell plates from an input stack and unloading of plates to the output stack. The microwell plates are covered with plastic lids to prevent evaporation. A high-precision gripper assembly provides a flexible a reliable solution to the transfer of plates from the input stack to the working position and from the working position to the output stack. The stacker allows the machine to run for about 6 hours unattended. The three pictures of the arrayer used are shown in Figure 6.
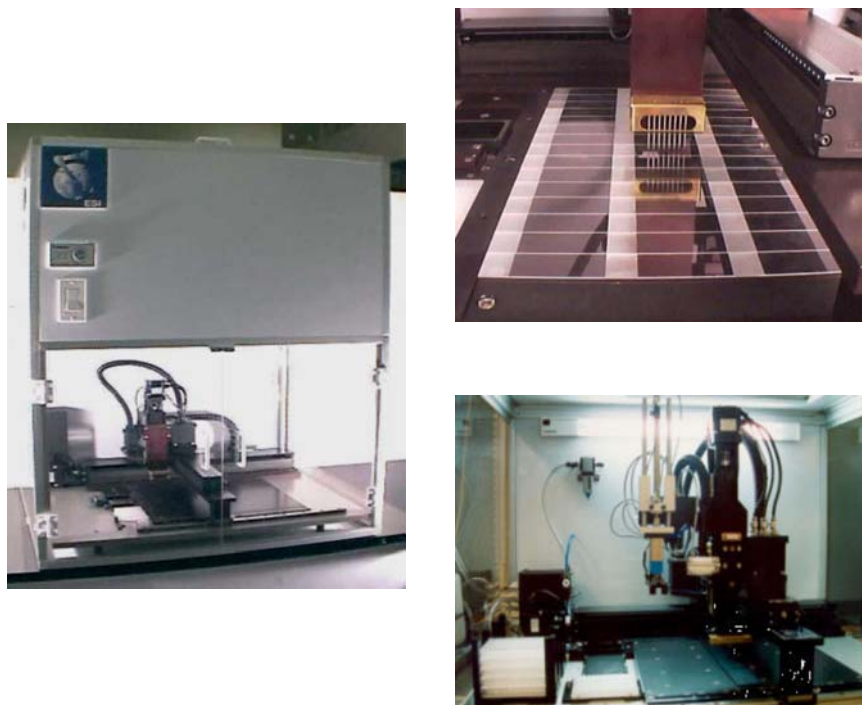
Figure 6: SDDC-2 Microarrayer. The general view of the arrayer (left), the printing on glass slides with the pins (right top), and the 384-well printing plates on the source plate holder (right bottom) (Virtek Vision Inc.).

2.2.7.2.3 Test prints

It is important to do test prints with the tips that will be used for the print run, immediately before beginning the run. A sample print plate, which is identical to the real print plates with 5 μl test print DNA or 1.5XSSC, was loaded and used for test printing onto slides from the same batch that will be printed on. The test print parameters (spacing and number of spots across) should match what the real array is going to have. Test prints must be done from different load positions on the 384-well plate to check the plate alignment, and on different slide positions on the platter to check the slide height adjustment on the platter. Because of the SSC, spots can be seen easily and even can be checked under the light microscope. Once everything is working perfectly on the test print, array print can be started.

2.2.7.2.4 Printing the real microarray

All the printing parameters (cycles of cleaning, sonication and drying times, total printing plates) were set properly. The dust and glass shards on the platter were sprayed off and the surface was wiped with moist kimwipe (paper towel). The slides were placed wearing powder free gloves, making sure they are aligned straight and lying flat. The magnets were put along the edges of the slides, so that slides will not move during the print run.

The spotting cDNAs in 384 well microplates were spun down just before putting on the plate holder. No liquids must be left on the sides of wells. The plates must be placed in the proper orientation. The spreadsheet listing the contents of each well in the printing plates must be ready on the computer. The order and orientation on the slide platter determines where each DNA is going to end up on the array.

When everything is ready, the real run can be started and monitoring is essential throughout the run to make sure all the tips are printing well on all the slides. The cDNA flows into the slit in the pin by capillary action, and is spotted on the slide by tapping down. Often, the first few slides have larger spots and if they begin running in to each other a lot, you can stop the run. This can be due to many problems (aging time of slides after poly-L-lysine coating, too much spotting DNA in wells, pin problems). When stopping and restarting the run for any reason, one must be sure that printing starts exactly where it stopped.

Once all plates are printed, all slides are kept in their position till the next day or at least 3 hours before numbering the slides on their right bottom side by using crystal pen.

2.2.7.2.5 Blocking slides

The glass slides also need to be treated after the printing process. First, the slides were UV-cross-linked. A dose of 450 mJ was applied with a Stratagene Stratalinker to bind the cDNA probes to the lysine coating.

To reduce non-specific binding of strongly negatively charged probe on microarray slides, the positively charged amine groups on poly-L-lysine coated slides are passivated by reaction with succinic anhydride. The slides were put in 25-30-slide rack, placed in a clean glass tank and incubated for 20-30 min by shaking in freshly prepared blocking solution to block the poly-L lysine background. Boiling water was prepared in a separate clean glass tank and the slides were dipped in it immediately for 1-2 min to denature the cDNAs on them. The slides were then removed from water, immersed in 95% ethanol for about 1 min (190 proof, AAPer Alcohol, USA) and centrifuged for 5 min in a low speed (1000 rpm) swinging holder centrifuge and then transferred to a clean plastic slide box immediately, leaving lid open overnight in a dust-free cabinet to dry slides. Finally, the slides were stored at room temperature up to 2 months.

2.2.8 Amplification of mRNA

Universal Human Reference RNA (Stratagene) and patients' RNA was amplified by two rounds of ds cDNA synthesis (Superscript II RT-PCR kit ,GibcoBRL) with a dT $_{(15)}$ -T7 primer, followed by T7 in vitro transcription (T7 Megascript kit, Ambion) and amplified RNA (aRNA) extraction (Wang *et al,.* 2000) by using Mastercycler Eppendorf Scientific, Inc. (NY, USA) thermal cycler.

Current protocols for the competitive binding method of hybridization with two species of RNA require 100 µg of RNA from each sample. This is a reasonable amount of RNA to obtain from cell lines. When working with human samples, often one can not obtain this much RNA, and even if one can do, this material can not usually be reaccessed for follow up experiments. Therefore, it is necessary to amplify the RNA while preserving the integrity of the message profile. RNA amplified by this method has been demonstrated to give results similar to those obtained with total RNA.

2.2.8.1 First and second strand cDNA synthesis

For first strand synthesis, 3 µg total RNA was mixed in 9 µl DEPC H_2O with 1µl (0.5µg/µl) oligo dT(15)-T7 primer, heated to 70^oC for 3 min and cooled to room temperature. Then, 4 µl 5X first strand buffer, 1 µl TS (template switch) oligoprimer (0.5µg/µl), 2 µl 0.1M DTT, 1 µl anti-RNase (20 U/µl), 2 µl 10mM dNTP and 2 µl Superscript II RNase H⁻ Reverse Transcriptase (200 U/µl) were added and incubated at 42oC for 90 min.

For second strand synthesis, 106 µl DEPC H_2O, 15 µl Advantage PCR buffer, 3 µl 10mM dNTP, 1 µl RNase H (2 U/µl) and 3 µl Advantage cDNA polymerase mix (50X) were added to the cDNA reaction tube and incubated at 37^oC for 5 min to digest mRNA, 94^oC for 2 min to denature, 65^oC for 1 min for specific priming, and 75^oC for 30 min for extension. These reagents were added by making a mastermix for multiple samples. The reaction was stopped with 7.5 µl 1M NaOH solution containing 2mM EDTA and by incubating at 65^oC for 10 min to inactivate enzyme.

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2.2.8.2 Double strand cDNA clean up

1 µl linear acrylamide (0.1 µg/µl) and 150 µl Phenol:Chloroform: Isoamylalcohol (25:24:1) were added to the sample and mixed well by pipeting. The slurry solution was transferred to ready Phase Lock Gel (PLG) tube (centrifuged at 13,000 rpm for 5 min before used) and centrifuged at 14,000 rpm for 5 min at room temperature in order to recover phenol free supernatant. Approximately 150 µl aqueous phase was transferred to RNase/DNase-free eppendorf tube and first 70 µl 7.5 M ammonium acetate and then 1 ml 100% EtOH (200 proof AAPer Alcohol, USA) were added and centrifuged at 14,000 rpm for 30 min at room temperature. The EtOH was removed carefully and the pellet was washed with 500 µl 100% EtOH and spun down at 14,000 rpm for 6 min. The supernatant was discarded by using a pipette, the pellet was air dried and resuspended in 60 µl DEPC H_2O.

Bio-6 chromatography column was prepared by washing with 700 µl DEPC H_2O 3 times to get rid of Tris and spun down at 3000 rpm for 2 min at room

temperature. The columns must be shaken well before draining to get rid of air bubbles. The gel in the column must become white and dry when ready to use. 60 μl sample was added to the centre of the column and centrifuged at 3000 rpm for 4 min. Then, the cDNA was dried in SpeedVac at high temperature for approximately 30 min and resuspended in 8 μl DEPC H$_2$O. The cDNA can be stored at –20°C if it will not be used directly for In Vitro Transcription (IVT)

2.2.8.3 In vitro transcription and aRNA purification

IVT reaction was performed using Ambion T7 Megascript kit. 2 μl of each 75 mM NTP (A, G, C, and UTP), 2 μl reaction buffer and 2 μl enzyme mix (RNase inhibitor and T7 phage polymerase) were added to the 8 μl cDNA sample, mixed well by pipeting and incubated at 37°C for 5-6 hours.

Amplified RNA (aRNA) was purified by using Trizol (GibcoBRL). 1 ml of Trizol solution was added to each IVT tube and mixed well. 200 μl chloroform was added per 1 ml Trizol solution, mixed well by shaking vigorously for 15 sec, incubated at room temperature for 2-3 min and centrifuged at 13,000 rpm for 15 min at 4°C. The aqueous phase was transferred to a new RNase free tube and 500 μl isopropyl alcohol was added per 1 ml Trizol reagent. The sample was mixed by inversion, incubated at room temperature for 10 min, and centrifuged at 14,000 rpm for 15 min. The pellet was washed 2 times with 75% EtOH in DEPC-treated water and centrifuged at 14,000 rpm for 5 min. The pellet was air-dried and quickly resuspended in 20 μl DEPC-treated water. The concentration and quality of aRNA was measured by spectrophotometer.

2.2.8.4 Second round amplification

1μg aRNA was mixed in 9 μl DEPC H$_2$O with 1μl random primer (2μg/μl), (GibcoBRL), heated to 70°C for 3 min and cooled to room temperature. Then, 4 μl 5X first strand buffer, 1 μl oligo dT-T7 primer (1μg/μl), 2 μl 0.1M DTT, 1 μl anti-RNase (20 U/μl), 2 μl 10mM dNTP and 2 μl Superscript II RNase H$^-$ Reverse

Transcriptase (200 U/μl) were added and incubated at 42°C for 90 min. From here, the above procedure of first round amplification for ds cDNA, clean up and IVT reactions were repeated.

Positive control RNAs (Spike controls)

Positive control RNAs were obtained by in vitro transcription, from poly(A) tail-modified bacterial gene constructs pGIBS-PHE, pGIBS-THR, pGIBS-TRP and pGIBS-DAP (American Type Culture Collection). Dr. Mathias Frevel kindly supplied these spike controls.

2.2.9 Preparation of probe

5 μg of aRNA from both test and reference RNA was used in the amino-allyl labeling reaction. The 2 μl dT_{18} primer (1 μg/μl), 1 μl anti-RNase (20 U/μl), 2.7 μl random primer (3μg/μl) and 1 μl spike controls (1 ng/μl) were added to 5 μg aRNA and the volume was brought to 15.5 μl with DEPC H_2O. The mixture was vortexed, spun down, incubated at 70°C for 10 min and placed on ice. Then, the reaction mix was assembled with 6 μl 5X first strand buffer, 0.6 μl 50X aadUTP/dNTP mix (500 μM each dATP, dCTP, dGTP, 300 μM aa-dUTP and 200 μM dTTP for 1 X dNTPs), 3 μl 0.1M DTT, 1.9 μl Superscript II RNase H⁻ Reverse Transcriptase (200 U/μl), 3 μl H_2O, vortexed, spun down and incubated at 42°C for 2 hours.

For RNA degradation, 10 μl 0.1M NaOH was added to each sample, incubated at 70°C for 10 min, and cooled to room temperature on bench for 5 min. Then, 10 μl 0.1M HCl was added per sample.

For EtOH precipitation, the solution was transferred to 1.5 ml eppendorf tube. 4 μl 3M NaAc and 1 μl glycogen (20 mg/ml) or 1 μl linear acrylamide (0.1 μg/μl) was added, vortexed and spun down. 100 μl ice-cold absolute EtOH was added, the tube was incubated at –20°C for 30 min and centrifuged for 15 min at 4°C. The supernatant was discarded and the pellet was washed with 500 μl ice-cold 75%

EtOH. After centrifugation for 15 min at 4°C, the supernatant was discarded and the pellet was dried in SpeedVac on high temperature for 2 min. Finally, the pellet was resuspended in 5 µl 0.1M sodium carbonate buffer (pH 9).

For dye coupling, 5 µl (30 nmoles) Cy5-NH ester-dUTP and 5 µl (30 nmoles) Cy3-NH ester-dUTP (Amersham Pharmacia Biotech, Uppsala, Sweden) dissolved in 45 µl DMSO was added for test and reference samples respectively, mixed by pipetting up and down, and incubated at room temperature in the dark for 1 hour.

Probe clean up was done either by using GFX columns (Amersham Pharmacia Biotech. Inc) or QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. For GFX columns, 90 µl H$_2$O was added per dye coupling reaction and columns were placed in a collection tube. 500 µl Capture Buffer was added to the column and the DNA solution was transferred. The mixture was mixed thoroughly by pipetting the sample up and down 4-6 times, and centrifuged at 13,000 rpm for 30 sec. After the first elution, the flow-through was put back into the column and centrifuged again. Then, the flow-through was discarded by emptying the collection tube and the GFX column was placed back inside the collection tube. 500 µl Wash Buffer was added and centrifuged at 13,000 rpm for 30 sec. Collection tube was discarded and GFX column was transferred to a fresh 1.5 ml microcentrifuge tube. 50 µl TE buffer (pH 8.0) was applied directly to the top of glass fiber matrix in the GFX column, incubated for 1 min at room temperature and centrifuged at 13,000 rpm for 1 min to recover purified DNA. For QIAquick PCR purification kit, 35 µl 100 mM NaOAc pH 5.2 and 500 µl PB buffer was added to per dye coupling reaction, applied to Qiaquick column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and 750 µl PE buffer was added and centrifuged again at 13,000 rpm for 1 min. After discarding the flow-through, the column was centrifuged at 13,000 rpm for 1 min and placed in a new tube. 50 µl EB buffer was applied directly onto the middle of the column, incubated for 1 min at room temperature and centrifuged at 13,000 rpm for 1 min to recover purified DNA.

Finally, for both probe clean up systems, the samples were concentrated by SpeedVac at high temperature for approximately 40 min until pretty much dry, resuspended in 14.23 µl H_2O and vortexed to ensure complete dissolving.

2.2.10 cDNA microarray hybridization

The volume required for hybridization is dependent on the size of array used. 20 µl volume was sufficient for test arrays containing 1500 targets by using a standard (24 x 20 mm) cover slip, however 35 µl hybridization volume was used for 10,750 targets using big (24 x 60 mm) cover slip.

After labeling reaction, the samples (control and patient) labeled with different dyes were combined together and denatured in hybridization solution (3.5 µl 20XSSC, 1.16 µl yeast tRNA (4 mg/ml) (Sigma), 1.16 µl polyd(A)$_{40-60}$ (8 mg/ml) (Amersham) and 0.7 µl 5% SDS) for 2 min at 95°C and cooled to 65°C before hybridization. Yeast tRNA and polyd(A)$_{40-60}$ were used to block the species. The slides (arrays) were prepared at least 1 hour before hybridization, by placing in hybridization-chamber, adding 30 µl hybridization-buffer (2XSSC/0.1%SDS) to reservoirs, closing hybridization-chamber and placing them in 65°C water bath. Then, the samples (35 µl) were placed on prewarmed slides and covered carefully with big cover slip. The hybridization was performed in a sealed, humidified corning chamber at 65°C overnight (16-18 hours) in submerged water bath.

2.2.11 cDNA microarray washing and scanning

The next day, the residual unbound probe was removed from slides by washing 1X for 10 min with 2XSSC/0.1%SDS, 1X for 10 min with 2XSSC (both prewarmed to 55°C) and 1X for 5 min with 0.2XSSC (room temperature). The final washing can be repeated if necessary. Air drying of slides after this step, frequently leaves a fluorescent haze on the slide surface. Because of this, before scanning, the slides were centrifuged in falcon tube for 5 min at 1000 rpm. The water used for preparation of buffers must be dd$H_2$0.

The scanning was performed with a GenePix 4000A laser-scanner (Axon Instruments) as soon as possible. The excitation was provided by individual 635 nm and 532 nm lasers. These wavelengths correspond to the ideal wavelengths used to excite the fluorophores Cy3 (excited by green laser light as 532nm) and Cy5 (excited by red laser light as 635nm) (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.2.12 Data normalization and analysis

Raw fluorescence data were acquired with GenePix 1.0 software (Axon). Laser settings were chosen to avoid signal saturation and achieve an overall median Cy3/Cy5 ratio of 0.8 to 1.2. The raw data was imported into GeneSpring software version 4.1.1 (Silicon Genetics). Each spot was defined by manual positioning of a grid of circles over the array image. Spots that were not suitable for accurate quantification, due to artifacts or low signal intensity, were excluded from further analysis. For each gene probe, the signal intensity ratio of patient over the control sample was calculated with raw fluorescent intensities, with the local background subtracted to determine net signal. The ratios were then normalized based on the distribution of all values with locally weighted polynomial regression (LOESS). That is to say, normalization was done for labeling efficiency, determined by scanner software based on the overall signal intensity (total intensity normalization) (Quackenbush, 2001). In order to recover reality, the ratios of each array were corrected so that the mean ratio of the four positive-control probes that had been spiked into every sample in a constant amount equaled 1.

Multiple image analysis (Gene clustering) was again done with GeneSpring software version 4.1.1 (Silicon Genetics, Redwood, CA). As some experiments work better than the others, it was important to use spots that were common in all experiments before cluster analysis. Because of this, 4,997 common data points that passed the restriction were used in gene clustering.

2.2.13 Expression analysis of a gene by semi-quantitative RT-PCR

Semi-quantitative RT-PCR (SRT-PCR) was performed to confirm the array result, as well as analyze the remaining samples by using the GeneAmp System 9600 (Perkin-Elmer, USA) thermal cycler.

2.2.13.1 First strand cDNA synthesis, fidelity and DNA contamination control

Equal amounts of original total RNA were used in cDNA syntheses performed by RevertAid First Strand cDNA synthesis kit (MBI-Fermentas, Vilnius, Lithuania). 3 µg of total RNA was mixed with 1 µl of oligo $(dT)_{18}$ primer ($0.5µg/µl$) and reaction volume was completed to 12 µl by using nuclease free deionized water, incubated at $70^{o}C$ for 5 min and chilled on ice. Then, 4 µl 5X reaction buffer, 1 µl ribonuclease inhibitor ($20U/µl$) and 2 µl 10 mM dNTP were added and incubated at $37^{o}C$ for 5 min. Following addition of 1 µl RevertAid M-MuLV reverse transcriptase ($200U/µl$), the mixture was incubated at $42^{o}C$ for 60 min. The reaction was stopped by heating at $70^{o}C$ for 10 min.

The quality of cDNA was initially tested by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR amplification using 1/40 volume of cDNA preparation. GAPDH primer pair was designed to produce a 151 bp fragment from cDNA and 250 bp fragment from genomic DNA, which enables detection of genomic DNA contamination in the RNA preparation.

2.2.13.2 Determination of optimal cycle of a gene for semi-quantitative PCR

To determine the total PCR cycle numbers that will define the logarithmic phase of amplification (optimum cycle not saturated for the amplified DNA fragment), an initial study was performed at 15 through 30 cycles for each primer pair. 80% of PCR product from each cycle was loaded on 2% agarose gel and

determined cycle number was used for amplification of the gene of interest in further studies.

2.2.13.3 *GAPDH* normalization and semi-quantitative RT-PCR

1/40 volume of first strand cDNA preparation was used for PCR amplification of *GAPDH* transcript using the pre-determined optimal cycle number (21 cycles) for *GAPDH*. 80% of all reactions were resolved by electrophoresis on 2 % agarose gel and visualized by ethidium bromide staining. Quantitation of relative band intensities was performed using Multi-Analyst software version 1.1 (Bio-Rad Laboratories). These intensities were used as reference for cDNA equalization with *GAPDH* amplification.

cDNA preparations yielding equal amounts of GAPDH products were used for further PCR studies (*IFITM1*, *CXCL3*, *CCNH*, *RAB1A*, *PRKAR2B*, *UCP2*, and *PTGS1*). All reactions were carried out under the following conditions: initial denaturation at 94°C for 3 min, optimum cycles of 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing), 72°C for 40 sec (extension) and a final extension at 72°C for 10 min. After the PCR cycles were completed, the tubes were held at 4°C for at least 5 min or until removal. 80% of all reactions were resolved by electrophoresis on 2 % agarose gel and visualized by ethidium bromide staining. Quantitation of relative band intensities was performed using Multi-Analyst software version 1.1 (Bio-Rad Laboratories). Each product was normalized against *GAPDH*.

The RT-PCR reactions were repeated several times. A pool of RNA from leukocytes of four healthy volunteers was used as a control sample.

2.2.14 Expression analysis of a gene by real-time RT-PCR

The real-time RT-PCR assays were done with the iCycler instrument (BioRad Laboratories) using LightCycler-DNA Master SYBR Green I (Roche, Molecular Biochemicals). The cDNA synthesis was done as mentioned above. The PCR

reactions were set up in a volume of 20 μl, containing 5 μl of sample cDNA (1:5 dilution of the RT reaction in nuclease free water), 1x SYBR Green I dye, 1.5 mM MgCl$_2$, and 5 pmoles from *IFITM1* and *GAPDH* specific primers.   The cycling conditions were as follows : 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 45 cycles with initial melting at 95°C for 5 min.

Relative expression levels were calculated using the PCR threshold cycle number (C$_T$) for each CML and control sample (both of which were normalized according to *GAPDH* mRNA for differences in amount of total RNA added to the reaction), using the formula $2^{-(\Delta C_T \text{ sample} - \Delta C_T \text{control})}$ (Pfaffl 2001 ; Livak *et al.,* 2001 ; Tanaka *et al.,* 2003). ΔC_T represents the difference in C$_T$ values between the target and *GAPDH* transcripts. RT-PCR was performed in duplicates for each sample and average C$_T$ values were calculated.

2.2.15 Statistical analysis

Mann-Whitney U test

Levels of gene transcripts between high and low-risk CML were compared using Mann-Whitney U test (SPSS software version 10.0 and Matlab 6 www.mathworks.com, mannwhit matlab routine, http://www.biol.ttu.edu/Strauss/ Matlab/matlab.htm) (Mann *et al.,* 1947). *P* values <0.05 were considered statistically significant.

Kaplan-Meier analysis

The goal of Kaplan-Meier analysis was to estimate a survival curve from a sample. Vertical axis represents estimated probability of survival for a hypothetical cohort.   In this study, overall survival of patients was done according to new prognostic score (NPS) and gene expressions using SPSS software version 10.0 at Ankara University, School of Medicine, Department of Hematology. *P* values <0.05 were considered statistically significant.

2.2.16 Polymerase chain reaction (PCR)

PCR is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA. There are three distinct events in PCR: template denaturation, primer annealing and DNA synthesis. Template DNA was denatured by heating the reaction to 95-96°C. After denaturation the primers were allowed to hybridize to their complementary single-stranded target sequences. The temperature of this step depends on the homology of the primers for the target sequence as well as the base composition of the oligonucleotides. The last step was the extension of the oligonucleotide primer by the thermostable polymerase. Traditionally, this step was carried out at 72°C. Usually, the larger the template, the longer the time required for a proper extension.

In general, 50-100 ng DNA was used as a template. A 50 μl PCR reaction contained 5 μl 10X PCR buffer (final concentration 1X PCR buffer), 1.5-3.0 mM MgCl$_2$, 200 μM dNTP, 10 pmol forward primer, 10 pmol reverse primer and 1 U Taq polymerase. The volume was adjusted to 50 μl by adding ddH$_2$O. MgCl$_2$ concentration was optimized for each primer pair by setting up a series of PCR reactions using a range of MgCl$_2$ concentrations.

2.2.17 PCR and restriction enzyme digestion

For restriction enzyme digestion, PCR was performed in a reaction volume of 25 μl which contained 2.5 μl 10X PCR buffer, 1.5 mM MgCl$_2$, 200 μM dNTP, 10 pmol forward primer (Y13F), 10 pmol reverse primer (Y13R), and 1 U Taq polymerase. In general, 50-100 ng DNA was used as a template. The volume was adjusted to 25 μl by adding ddH$_2$O. Amplification was performed in the GeneAmp PCR with the following parameters: initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 sec (denaturation), 57°C for 30 sec (annealing), 72°C for 30 sec (extension); and a final extension at 72°C for 10 min. After the PCR cycles were completed, the tubes were held at 4°C for at least 5 minutes or until removal.

Restriction enzyme digestion with *Bgl* II (the recognition site is 5'-A▼GATCT-3') was performed in 20 µl reaction volumes using the reaction buffer recommended by the manufacturer. One unit of enzyme was sufficient to digest approximately 30-50 ng PCR product. The PCR template was quantitated by agarose gel electrophoresis using a DNA size marker of known concentration. In general, 5-10 µl PCR product was used. The incubation temperature was $37^{o}C$ for 2 hours. After digestion, heat inactivation was performed at $65^{o}C$ for 10 min. After incubation, the cut and uncut PCR fragments were analyzed by agarose gel electrophoresis (2%).

2.2.18 PCR and amplification refractory mutation system (ARMS)

In this PCR-based technique, one of the PCR primers precisely matches one allelic variant of the target sequence, but it is mismatched to the other. Two PCR reactions were performed for each sample. One reaction contained the specific primer (165N) for the normal allele and the other (165M) for the mutant allele. Both reactions had a common primer (165C). PCR reactions were carried out in a total of 25 µl which contained 2.5 µl 10X PCR buffer, 3 mM $MgCl_2$, 200 µM dNTP, 20 pmol from each primer, 50-100 ng genomic DNA and 1 U Taq polymerase. The volume was adjusted to 25 µl by adding ddH_2O. Amplification was performed in the GeneAmp PCR with the following parameters: initial denaturation at $95^{o}C$ for 3 min; 30 cycles of $95^{o}C$ for 30 sec (denaturation), $62^{o}C$ for 30 sec (annealing), $72^{o}C$ for 30 sec (extension); and a final extension at $72^{o}C$ for 10 min. After the PCR cycles were completed, the tubes were held at $4^{o}C$ for at least 5 minutes or until removal.

CHAPTER 3. RESULTS

3.1 Patient information

Patient information with NPS calculated is given in Table 2. NPS for low-risk (n=39, NPS:21→708), intermediate-risk (n=12, NPS:784→1379), and high-risk (n=16, NPS:1483→3853) CML are ≤ 780 with median survival time of 98 months, > 780 and ≤ 1480 with median survival time of 65 months, and > 1480 with median survival time of 42 months, respectively. Patients ranged in age from eighteen to eighty years old, with a mean age of 43.4±13.2 (mean±SD), and a male to female ratio of thirty-one to thirty-six. Regardless of risk group assignment, all patients received a short course of hydroxyurea followed by hydroxyurea or interferon during the median follow up duration of 26.5 months. Six patients who did not achieve remission with interferon based on BCR-ABL fusion analysis, received imatinib mesilate sequentially. Four patients (two low, one intermediate and one high-risk) received stem cell transplantation from siblings and are still alive.

Table 2: Patient samples with NPS values

RNA Code	Age at Diagnosis	New Prognostic Score (NPS)
High Risk (n=16)		
CML-2	31	1494,6
CML-3	45	1510,8
CML-4	42	1968,3
CML-7	22	3853,2
CML-10	50	1782,7
CML-15	29	1483,7
CML-23	28	1671,6
CML-32	38	2549,9
CML-45	56	1513,4
CML-55	53	1604,1
CML-58	52	1514,1
CML-64	20	1518,2

CML-71	50	1496,4
CML-87	51	1511,2
CML-89	55	1617,2
CML-91	37	1498,4

Low Risk (n=39)

CML-6	24	141,8
CML-8	35	583,9
CML-12	52	707,9
CML-13	40	578,2
CML-14	46	382,9
CML-17	63	707,9
CML-18	25	41,3
CML-22	36	634,5
CML-25	43	259,4
CML-29	36	258,5
CML-31	47	21
CML-46	39	20,7
CML-51	38	24,8
CML-54	42	141,4
CML-56	46	111,5
CML-59	32	204
CML-60	65	667
CML-62	35	496,5
CML-65	47	204
CML-66	18	21
CML-67	32	210
CML-68	37	210
CML-69	24	550,4
CML-70	58	667
CML-74	23	141
CML-75	34	214
CML-76	28	102,4
CML-77	34	118,1
CML-78	49	163,5
CML-79	26	20,6
CML-80	33	41,7
CML-81	38	217,3
CML-82	25	104,8
CML-83	51	707,6
CML-84	43	576,2
CML-85	47	258,5
CML-86	29	284,1
CML-88	54	624
CML-90	35	43,1

Intermediate Risk (n=12)

CML-9	43	829,8
CML-11	70	1024,7

CML-19	51	1237,7
CML-20	51	1309,6
CML-28	57	1168,6
CML-30	66	1378,6
CML-38	54	783.6
CML-43	42	1101,9
CML-53	29	841,7
CML-63	69	1017
CML-72	80	912
CML-73	53	1079,6

3.2 Test array experiment with K562 cell line

The aim of this experiment was to optimize the amount of amplified RNA (aRNA) that would be used in microarray experiments and to test the printed arrays that had known interferon stimulated genes (ISGs) spotted on the glass slides.

Two sets of K562 cells were plated at 2 x 10^6 cells/ml in 50 ml of medium overnight and one of them was treated with 1,000 U/ml interferon α-2b (IFNα-2b) for 16 hours. The other plate was left untreated and used as a control. Total RNA was isolated and amplified. 1, 3, 5, 10 and 15 μg aRNA from treated and untreated samples were labeled with Cy5-NH ester-dUTP and Cy3-NH ester-dUTP, respectively. 5 μg aRNA was found to give better results on arrays and therefore, the same amount was used for CML samples in future experiments.

One part of this array is shown in Figure 7 and the two red spots indicated by the arrow, represent G1P3 interferon, alpha-inducible protein (clone IFI-6-16) (Hs.265827). This gene is induced approximately 16-fold in the treated sample with IFNα-2b, compared to untreated sample. The list of genes at least 2-fold induced is given in Table 3. The down-regulated genes were not observed in this particular experiment as expected.
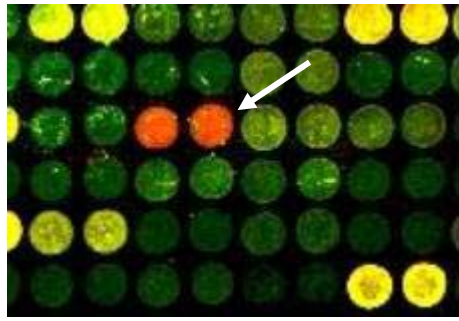
Figure 7: K562 cell line array picture. The red spots indicate the gene *IFI-6-16.*

Table 3: Genes at least 2 fold induced by IFNα-2b in K562 cell line

Unigene ID	Gene Name	Fold Induction
Hs.265827	interferon,alpha-inducible protein	16
Hs.77225	ADP-ribosyltransferase	11
Hs.5947	mel transforming oncogene	8
Hs.840	indoleamine-pyrrole 2,3 dioxygenase	7
Hs.833	interferon-stimulated protein, 15kDa	5.5
Hs.146360	interferon-induced transmembrane protein	3
Hs.170040	platelet-derived growth factor receptor	2.3

3.3 Array results of CML samples

Problems with the samples

All CML samples were prepared, stored and processed in the same way. However, most of the samples did not work on the arrays. There was no obvious problem in the RNA extraction and amplification.

Current protocols for the competitive binding method of hybridization with two species of RNA require at least 100 μg of total RNA from each sample. This is a reasonable amount of RNA to obtain when working with cell lines. However, with human samples, often one cannot obtain this much RNA, and even if having the

enough amounts, this material cannot usually be reaccessed for follow up experiments. Therefore, it was necessary to amplify the RNA while preserving the integrity of the message profile. The amplification protocol used involved two rounds of double stranded cDNA synthesis followed by in vitro transcription (IVT). RNA amplified by this method has been demonstrated to give results similar to those obtained with total RNA (Wang *et al.,* 2000).

The quality of the RNA in labeling reactions has a marked effect on the quality of the hybridization. RNA preparations, which look good by the standard molecular biology criteria, can give poor results. Because of this, many optimization experiments must be performed to standardize the protocols.

In some cases, after the labeled probe clean up, the samples produced a gelatinous precipitate that was recovered in the concentrated volume. The presence of this material, signals the presence of contaminants. The more extreme the contamination is, the greater the fraction of probe, which will be captured in this gel. Even if heat solubilized, this material tends to produce uniform, non-specific binding to the DNA targets.

To get rid of the possible contaminants and carbohydrates, Lithium chloride (LiCl) precipitation solution was used in some CML samples for trial, which is an effective way. Then, the amplification and labeling reactions were repeated. However, the gelatinous precipitate was still obvious. To treat all the CML samples the same way, LiCl precipitation was not performed further in order not to lose the RNA samples.

Most probably, one problem of the samples resulted from preparation of buffy coat. For example washing it with 1xPBS affected the quality of RNA in most of the samples. Because, washing cells before TRIzol extraction, is not preferred as this increases the possibility of mRNA degradation.

Collection of whole blood is the first step in many molecular assays used to study cellular RNA. However, the major problem in such experiments is the instability of the cellular RNA profile in vitro. A method that preserves the RNA

expression profile during and immediately after blood is drawn is therefore essential for accurate analysis of gene expression in human whole blood. Nowadays, PAXgene Blood RNA System (Qiagen) is available for RNA isolation from blood. This system enables the collection, stabilization and transportation of whole blood specimens, together with a rapid and efficient protocol for isolation of cellular RNA. The system allows researchers to obtain a gene expression profile that is closer to the patient's true profile, facilitating studies of gene transcription in disease and drug monitoring. It was tried on arrays and gave very good results (www. preanalytix.com).

Poor array results of CML samples

The following figures are some examples for poor array results. These were not analyzed in detailed or the good parts of the array were analyzed in some cases, excluding artifacts.

In Figure 8, there is a high background over the entire hybridized surface. This may be a result of contaminants in the samples, such as carbohydrates. Excessive washing of arrays with SSC tends to minimize the noise but not completely solves the problem.
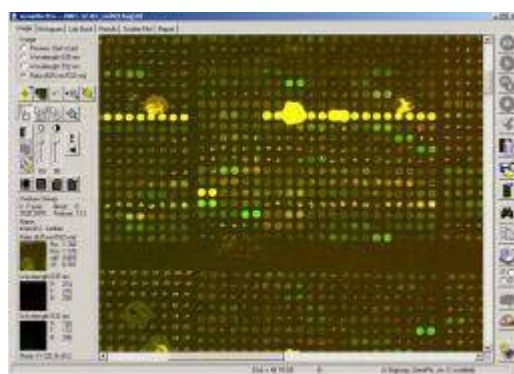
Figure 8: Array with high background.

In Figure 9, the array looks fine, however, there is a scratch on the right side, most probably due to cover slip friction problem during washing steps.
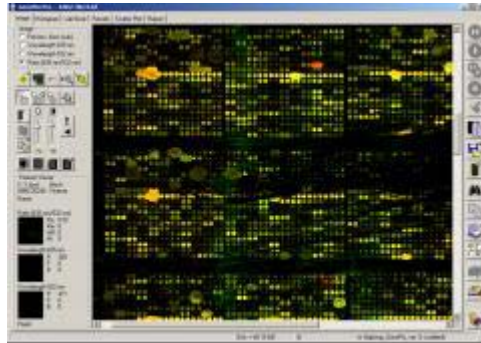
Figure 9: Array with scratches.

Cy5 labeling that did not work for a CML sample is shown in Figure 10. Since, spike controls worked for both labeling reactions, the problem is due to the sample.
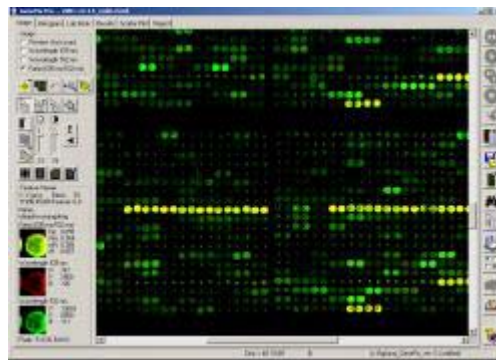
Figure 10: Array with Cy5 labeling problem.

Some of the spots are running into each other in Figure 11. This can be due to excess amount of the cDNA spotted in the 384 well plates and/or the aging problem of the slide after poly-L-lysine coating and/or pin problems of the arrayer.
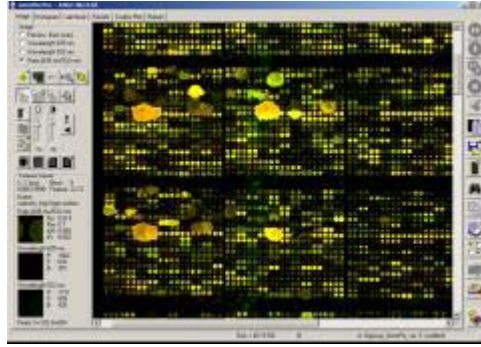
Figure 11: Array with spots running into each other.

In Figure 12, only the spike controls worked. Because of this, labeling reactions are fine, however both samples are not working. In this experiment, control sample was obtained from four volunteered healthy individuals.
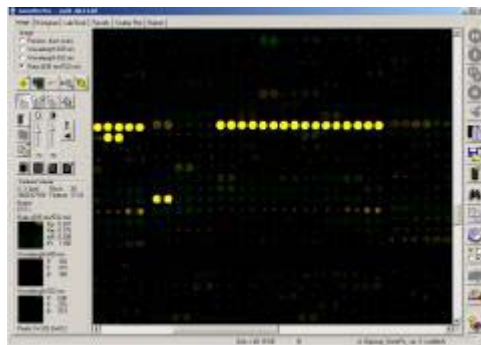
Figure 12: Array with not working samples.

There is a non-specific fluorescent background at the corner of the slide in Figure 13. This can be a result of air-drying of the slides after washing which frequently leaves a fluorescent haze on the slide surface. More likely, this background can be formed after blocking the slides. Because, when scanned after printing and before blocking, the slides look clean. However, after blocking, this fluorescent noise sometimes appears.
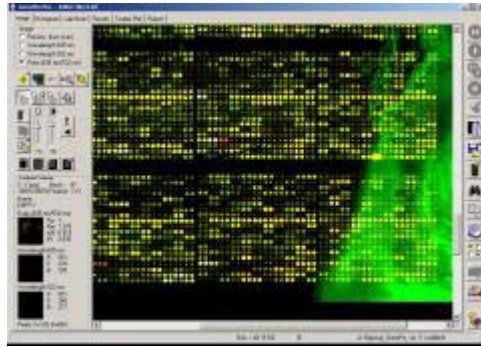
Figure 13: Array with fluorescent background.

Good array results of CML samples

Unfortunately, most of the CML samples did not work on the arrays, probably because of the total RNA quality that was poor for arrays. However, CML 2, CML 4, CML 8 and CML 12, which were not selected on purpose, worked very well.

Figure 14, 15, 16 and 17 show the array pictures of the CML 2, 4, 8, and 12 respectively. The red spots indicate that the test cDNA for that gene is more abundant than the reference cDNA, which means that it is being expressed at a level higher than the reference cDNA and a green spots indicates that the test cDNA is being expressed at a level lower than the reference cDNA. The yellow spots mean that there is no change in the expression level between the two population of test and reference cDNA.
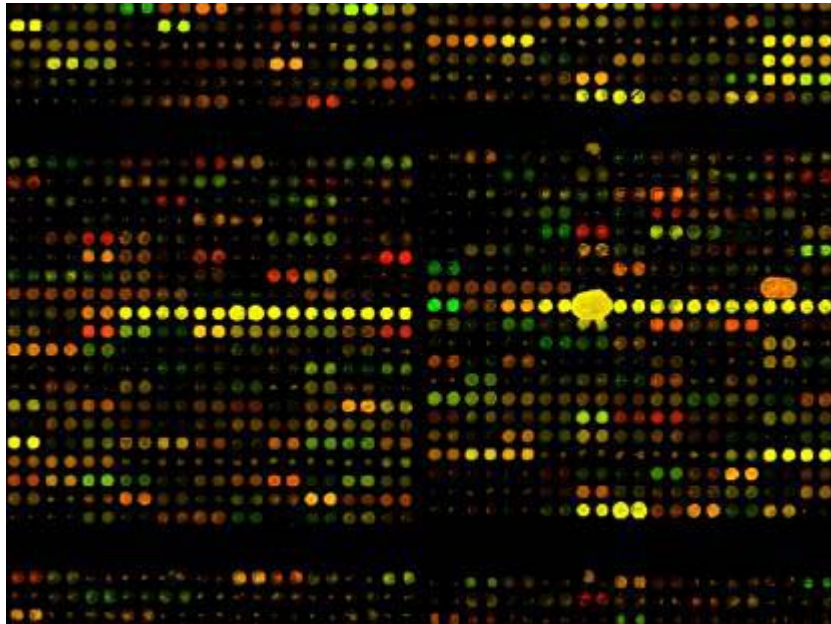
Figure 14: Array of CML 2 versus reference RNA.
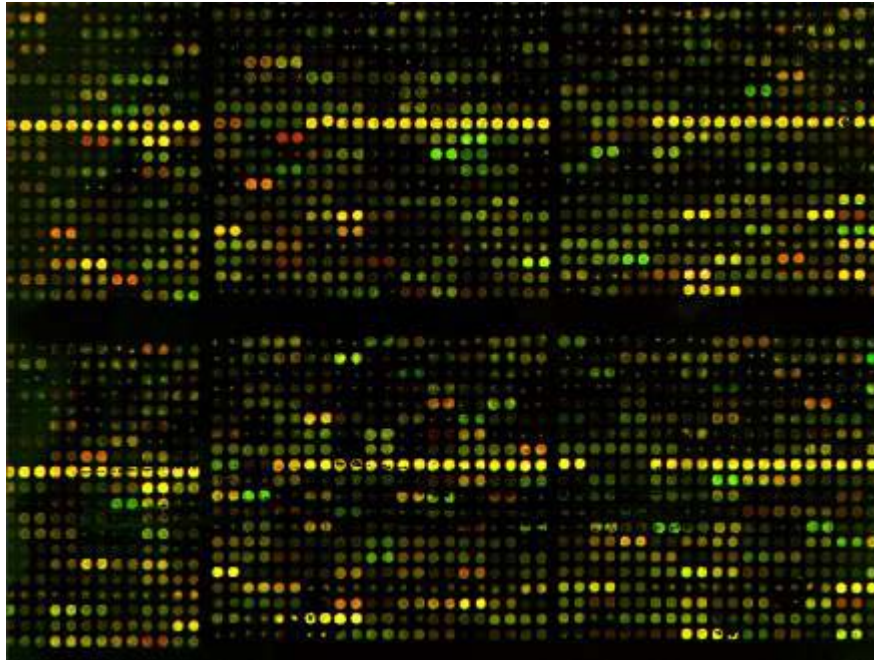
Figure 15: Array of CML 4 versus reference RNA.
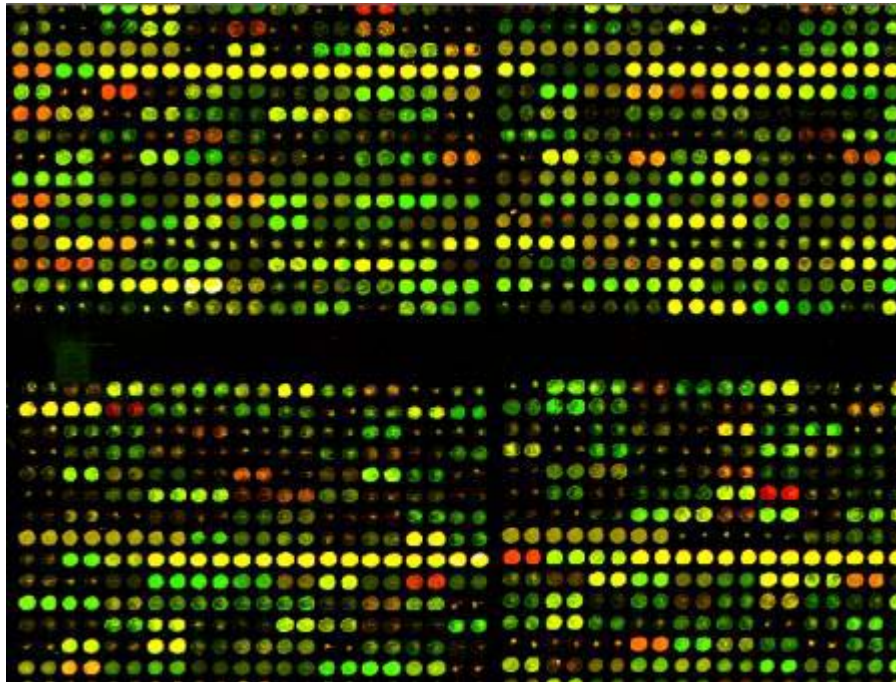
Figure 16: Array of CML 8 versus reference RNA.
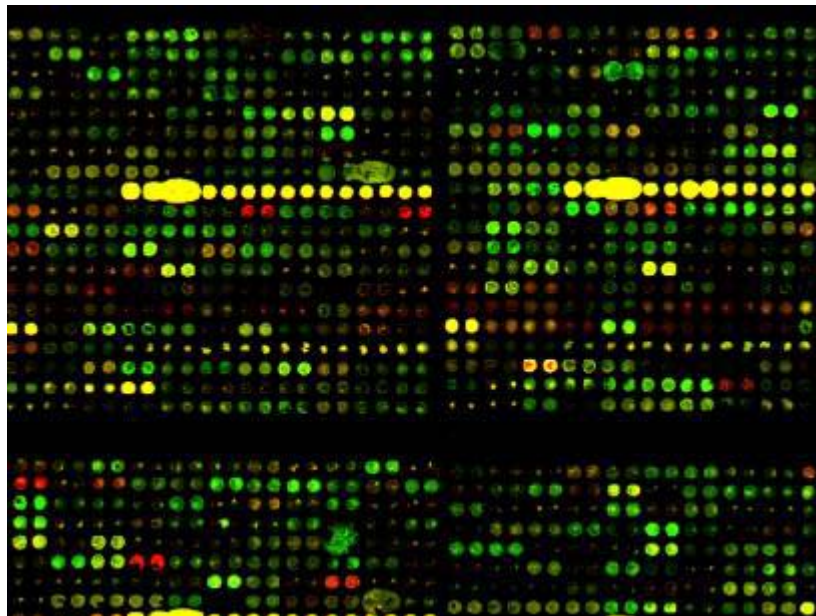
Figure 17: Array of CML 12 versus reference RNA.

The scatter plot view of CML samples

The scatter plot view is useful for examining the expression levels of genes in two distinct conditions, samples, or normalization schemes. For instance, you can use the scatter plot to identify genes that are differentially expressed in one sample versus another. A scatter plot can also be used to compare two values associated with genes in two gene lists.

In any scatter plot view, each `+' symbol represents a gene. The vertical position of each gene represents its expression level in the current condition, and the horizontal position represents its control strength (the median expression level of this gene). Thus, genes that fall above the diagonal are overexpressed and genes that fall below the diagonal are underexpressed as compared to their median expression level over the course of the experiment.

The scatter plots of CML experiments are given in Figure 18, 19, 20 and 21 respectively, for CML 2, 4, 8, and 12. The raw fluorescent data plotted onto scatter plot was obtained from GenePix scanner software and imported into the GeneSpring software version 4.1.1 for analysis. The Cy3 labeled reference is plotted on the x-axis and Cy5 labeled CML samples are plotted on the y-axis.
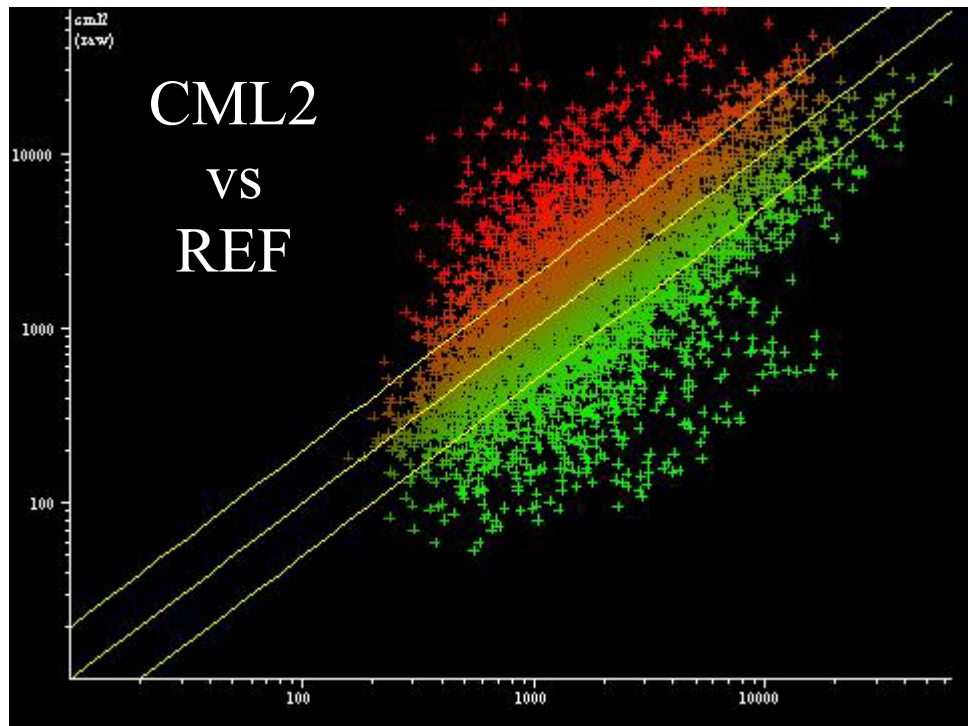
Figure 18: Scatter plot of CML 2 versus reference. Cy3 is on the x-axis and Cy5 is on the y-axis.
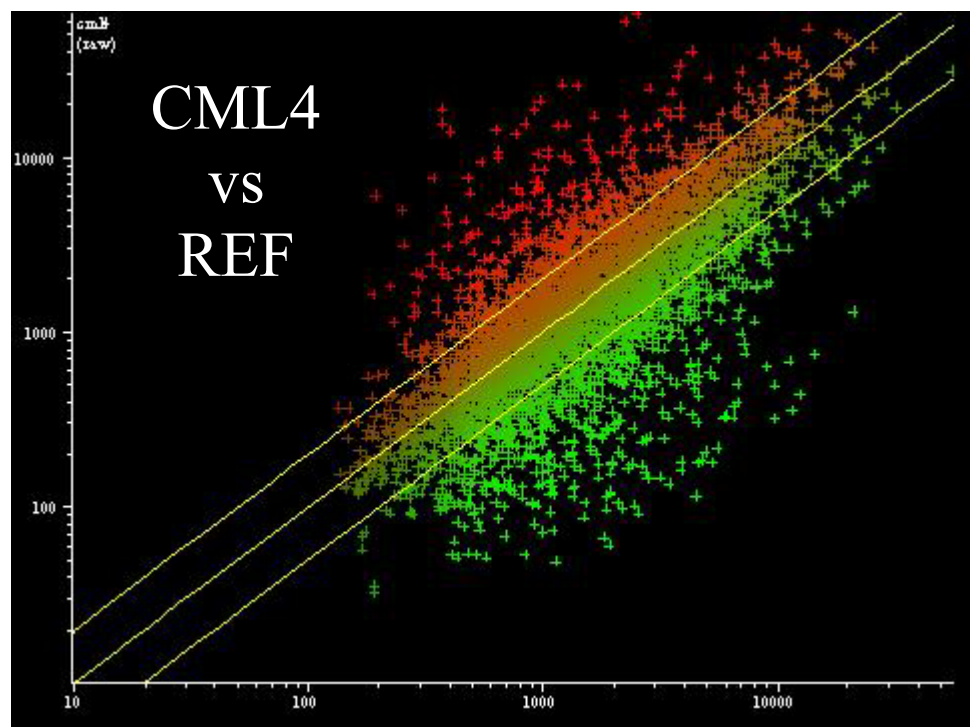
Figure 19: Scatter plot of CML 4 versus reference. Cy3 is on the x-axis and Cy5 is on the y-axis.
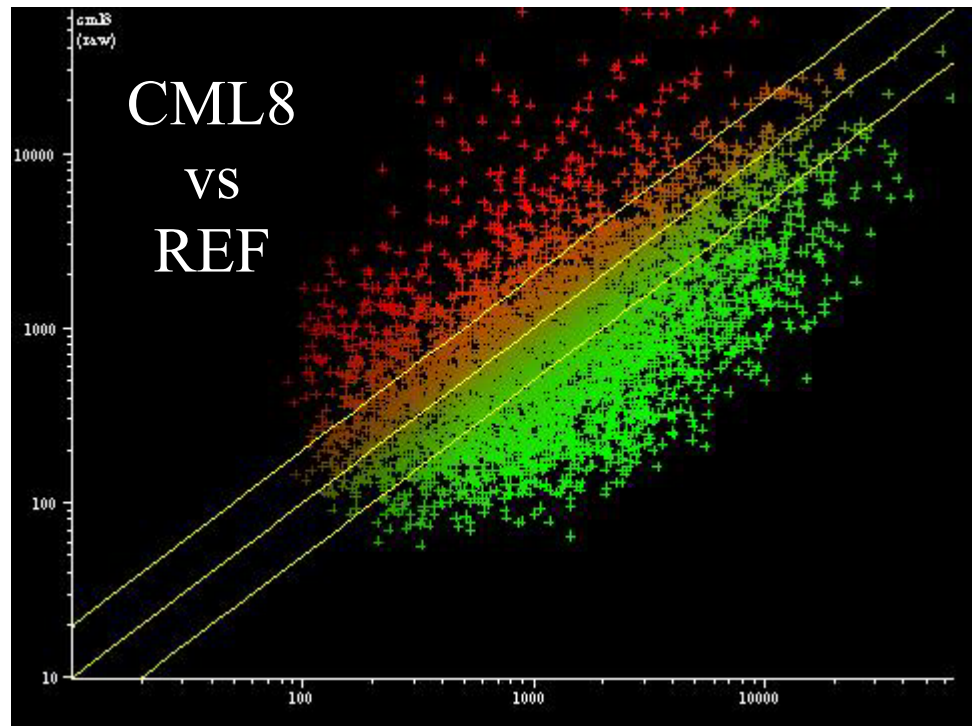
Figure 20: Scatter plot of CML 8 versus reference. Cy3 is on the x-axis and Cy5 is on the y-axis.
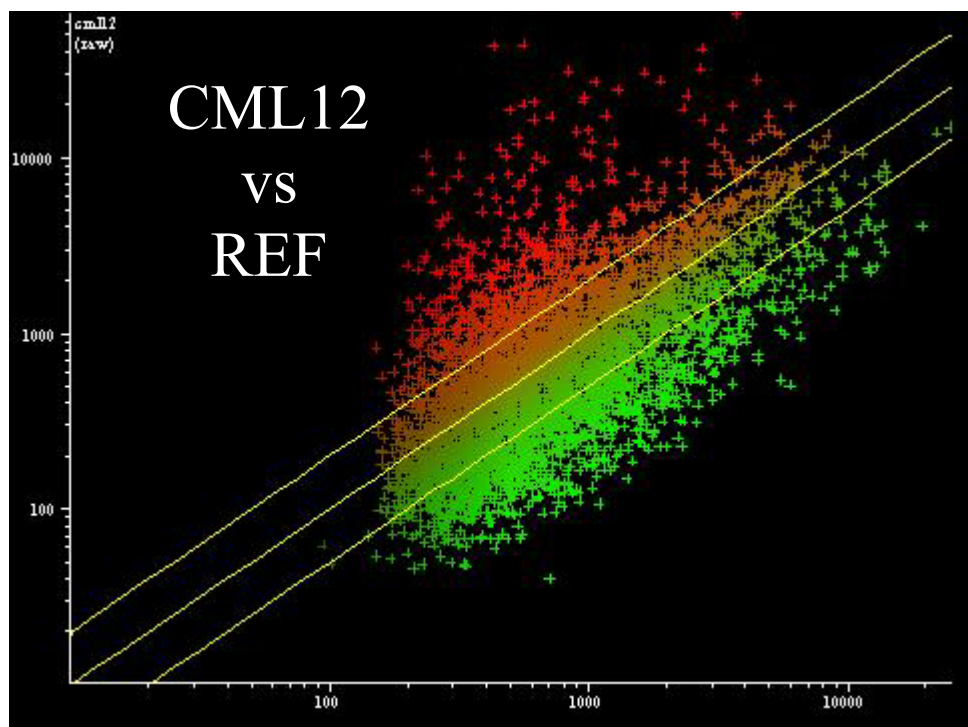
Figure 21: Scatter plot of CML 12 versus reference. Cy3 is on the x-axis and Cy5 is on the y-axis.

3.4 Cluster analysis of two high and two low-risk samples

Since some experiments work better than the others, it is important to include common spots while performing cluster analysis. Spots that were not suitable for accurate quantification, due to artifacts or low signal intensity, were excluded from further analysis, resulting in 4,997 common data points between all four experiments. In Figure 22, cluster of four samples are shown. The same reference cDNA was used in each experiment. In this image, the red colored genes indicate that they are more expressed than the green colored genes in each experiment. Differentially expressed genes are obvious, indicating the potential risk group indicator genes.
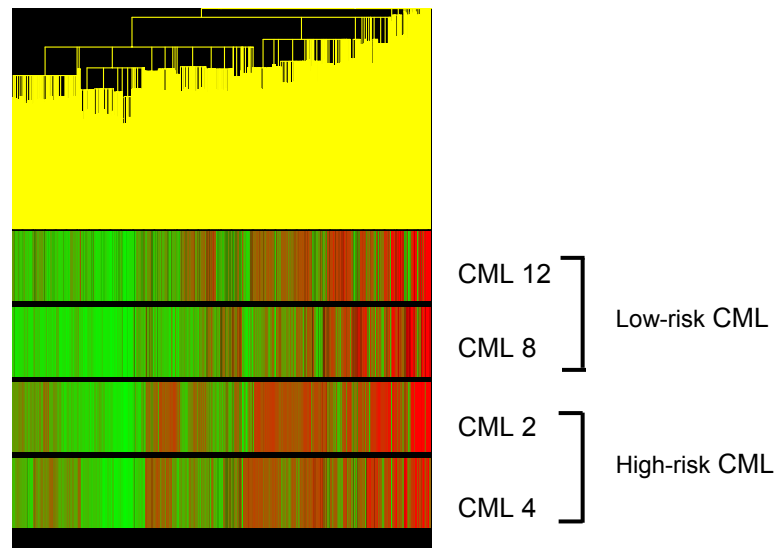
Figure 22: Gene clusters of four CML samples. The top two are the low-risk samples and the bottom two are the high-risk samples.

Figure 23 shows the gene clusters of four CML samples after zooming one specific part. The potential risk group indicator genes are more obvious in this image.
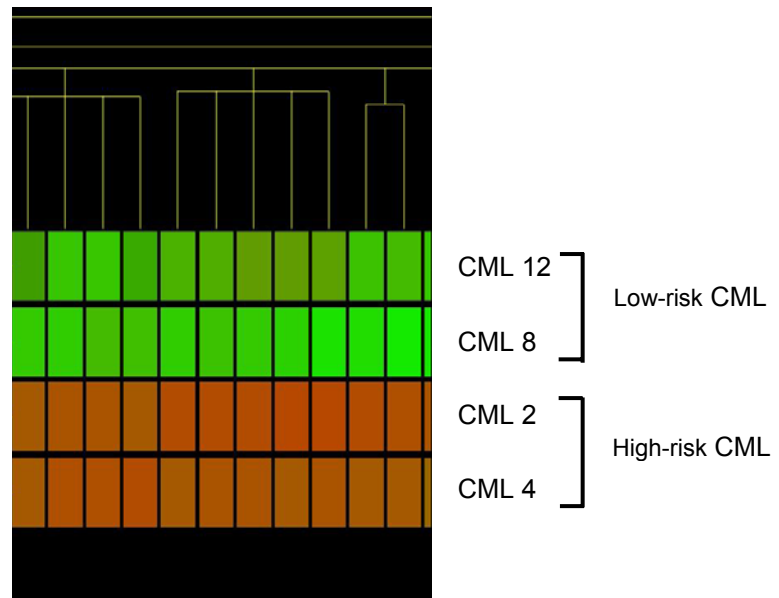
Figure 23: Zoomed gene cluster images of four CML samples. The potential risk group indicator genes are more obvious.

Differentially expressed genes identified by cluster analysis of the high (CML 2 and CML 4) and low (CML 8 and CML 12) risk CML patients are shown in Table 4. 58 genes were found to be expressed at least 3-fold higher in both high-risk samples compared to both low-risk samples and 25 genes were found to be expressed at least 3-fold higher in both low-risk samples compared to both high-risk samples. The top eleven differentially expressed genes in high-risk and low-risk samples are given in Table 5 and 6 respectively with fold inductions. The raw fluorescent data obtained from the patient samples and the controls are given in Appendix (c).

Table 4: Differentially expressed transcripts in high and low-risk CML patients

a. 58 transcripts expressed at least 3-fold higher in high-risk compared to low-risk samples.

Accession	Gene symbol	Function
AA279147	CSF2R	hematopoietic cell activation
AA181500*	PRKAR2B	signaling
W47101*	IL1B	thymocyte proliferation
R06438	?	unknown
N70773	DDEF2	regulator of cell growth and cytoskeletal organization
T74192	PROS1	anticoagulant plasma protein
N22684	DDX17	RNA dependent ATPase
AA935273	CXCL3	CXC chemokine
AA454668	PTGS1	proliferation
AA482286	SEL1L	notch signaling
AA707321/W86182*	PNN	adhesion, desmosome associated
N73222	MGP	inhibitor of bone formation
AI026973	HGD	homogentisate oxidation
AA463544*	MCP	membrane protein
R36587	PPI5PIV	unknown
AA913804	RIPK2	CASP-8-mediated apoptosis. NF-kappaB activation.
W42723	CXCL1	CXC chemokine
N78902	LEPR	leptin receptor
N24824	KIT	stem cell factor receptor
AA456869	RCOR	unknown
AA425238	RUNX1	transcription, acute myeloid leukemia 1 protein
AA453593	ATE1	arginilation
AA287107	ZNF302	transcription
H54020	SFRS7	splicing factor
R92806	GDI2	GDP/GTP exchange reaction regulator of RAB proteins
AA127116*	HNRPA1	RNA packaging and transport
T71308	CYP2C8	electron transport, principal enzyme of Taxol metabolism
R20770	STXBP3	structural protein
AA044390	UGP2	glucosyl donor in cellular metabolic pathways
N75017*	RPS6	selective translation of particular classes of mRNA
AA489232	APT6M8-9	acidification of a variety of intracellular compartments
R27581	CUL3	cell proliferation control
NM_001959	EEF1B2	eukaryotic translation elongation factor
AA019511	HMGB2	chromosomal protein, unwinds dsDNA
AA460930	TCEB1	regulatory subunit of the transcription elongation factor B
H50229	PP	metabolism
AW075605	RPL9	ribosomal protein
NM_003750	EIF3S10	translation factor
R93829	NAP1L1	modulation of chromatin formation and regulation of cell proliferation
N47717	FABP5	keratinocyte differentiation
AA454146*	CCNH	cell cycle control, transcription and DNA repair
AA448814	APG3	unknown
AA412053	CD9	platelet activation and aggregation
AA476508	ENPP2	potent tumor cell motility-stimulating activity
AA465603	MGC14433	unknown
N69689	RAB1A	protein transport
AA460981	GOLGA4	vesicular transport

Accession	Gene symbol	Function
N67039	?	unknown
AA099383	WRB	potential transcription factor
N59851	WASF1	signaling, thyrosine kinase receptors and small GTPases
AA446819	OAT	proline and ornithine metabolic pathway
R52654*	HCS	mitochondrial electron transport
AA598526*	HIF1A	homeostasis; angiogenesis, erythropoïesis
AA424824	DSTN	actin depolymerizing protein
AA406332	SEC23A	secretory, plasma membrane, and vacuolar protein transport
AW004895	HSPD1	chaperon; mitochondrial protein import and macromolecular assembly
AA001745	DC2	unknown
N93924	RFC4	DNA replication

b. 25 transcripts expressed at least 3-fold higher in low-risk compared to high-risk samples.

Accession	Gene symbol	Function
AA630549*	HLA-DRB1	MHC
T63324*	HLA-DQA1	MHC
AA634006*	ACTA2	cell motility
H08749	MAP2K3	signaling
AA436459	*NFIX*	transcription and replication
H61243	UCP2	mitochondrial protein transport
AI871056	S100A4	Calcium binding
AA931043*	HAGH	metabolism
T83159	LSP1	unknown
T58146	P5-1	MHC
AA599177	CST3	cysteine proteinase inhibitor
AA933862	CD3E	signaling
AI253411	ELAVL2	RNA binding
AA620426	AMPH	synaptic vesicle endocytosis
AA419251	IFITM1	IFN signaling
N69335*	COL9A1	structural protein
AA480071	TCF7	transcription, t-cell lymphocyte differentiation
NM_002168	IDH2	intermediary metabolism and energy production
AA458965	NK4	lymphocyte activation
T47442	PROCR	coagulation control
AA444009	GAA	metabolism
AA630328	LGALS3	galactose-specific lectin
AI675889	NPY	control of feeding
AA838691	EPHX1	metabolism
AI208702	MAP4K3	signaling

*Genes recovered multiple times on different spots on the array.

Table 5: The top eleven differentially expressed genes in high-risk compared to low-risk samples

Gene name	Gene symbol	Fold induction
Development and differentiation enhancing factor 2	DDEF2	17.0
Heat shock 60kd protein 1	HSPD1	12.0
Protein kinase, cAMP dependent regulatory, type ii beta	PRKAR2B	11.0
Pinin, desmosome associated protein	PNN	10.5
Interleukin 1, beta	IL1B	10.0
Chemokine C-X-C motif ligand 3	CXCL3	9.3
Membrane cofactor protein	MCP	8.7
Cytochrome c	HCS	8.3
Dead/h (asp-glu-ala-asp/his) box polypeptide 17 (72kd)	DDX17	8.0
Receptor-interacting serine threonine kinase 2	RIPK2	8.0
Chemokine C-X-C motif ligand 1	CXCL1	7.0

Table 6: The top eleven differentially expressed genes in low-risk compared to high-risk samples

Gene name	Gene symbol	Fold induction
Hydroxyacyl glutathione hydrolase	HAGH	17.7
Natural killer cell transcript 4	NK4	17.2
Major histocompatibility complex, class ii, dr beta 1	HLA-DRB1	15.8
Lectin, galactoside-binding 3	LGALS3	10.6
Major histocompatibility complex, class ii, dq alpha 1	HLA-DQA1	10.5
Isocitrate dehydrogenase 2	IDH2	8.7
Interferon induced transmembrane protein 1 (9-27)	IFITM1	8.5
S100 calcium-binding protein a4	S100A4	8.3
Transcription factor 7, t-cell specific	TCF7	8.2
Mitogen-activated protein kinase kinase 3	MAP2K3	6.0
Epoxide hydrolase 1	EPHX1	6.0

The scatter plots of 58 risk group indicator genes that were found to be expressed higher in high-risk samples are given in Figure 24, 25, 26 and 27 respectively, for CML 2, 4, 8, and 12. This appearance confirms the higher expression of these genes in high-risk CML samples, 2 and 4, because, almost all genes are above the cutoff line, compared to CML samples 8 and 12.
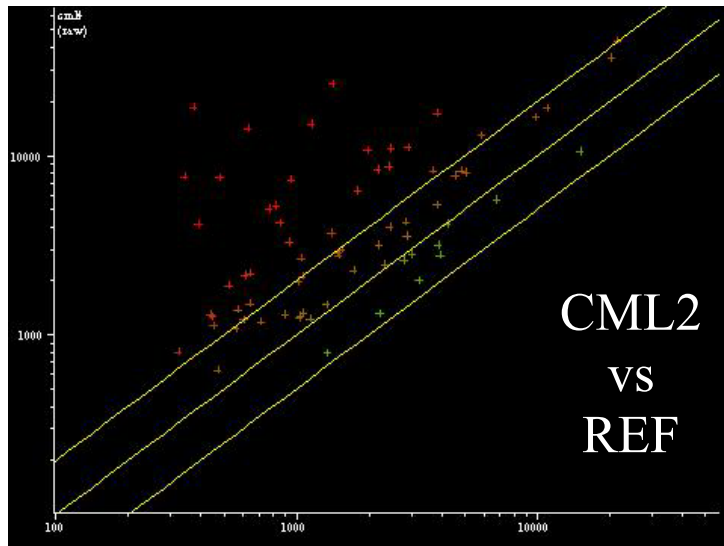
Figure 24: Scatter plot of 58 risk group indicator genes in CML 2. These genes were found to be expressed at least 3-fold higher in high-risk samples. In this figure, CML 2 is on the y-axis and the reference is on the x-axis.
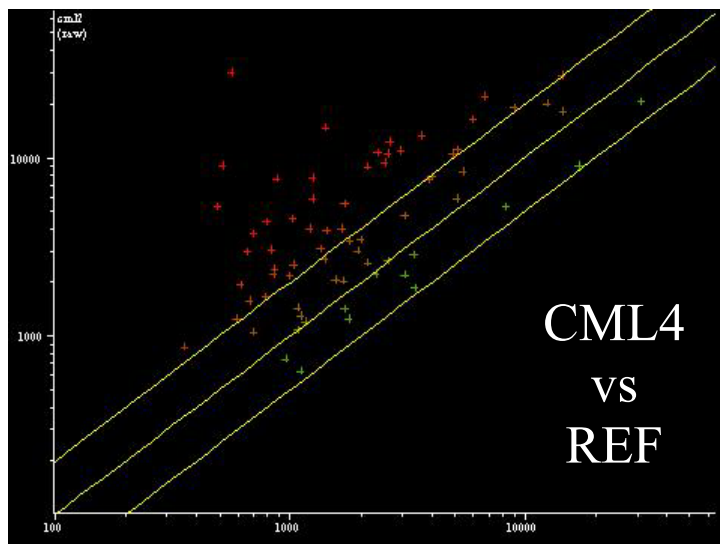
Figure 25: Scatter plot of 58 risk group indicator genes in CML 4. These genes were found to be expressed at least 3-fold higher in high-risk samples. In this figure, CML 4 is on the y-axis and the reference is on the x-axis.
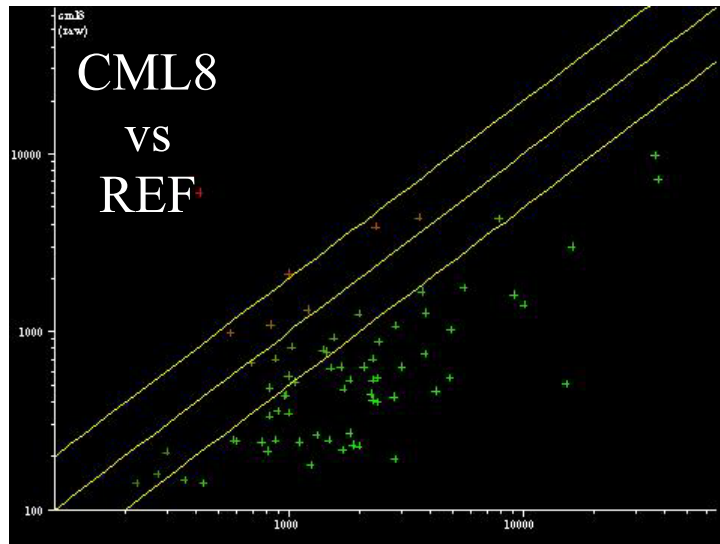
Figure 26: Scatter plot of 58 risk group indicator genes in CML 8. These genes were found to be expressed at least 3-fold higher in high-risk samples. In this figure, CML 8 is on the y-axis and the reference is on the x-axis.
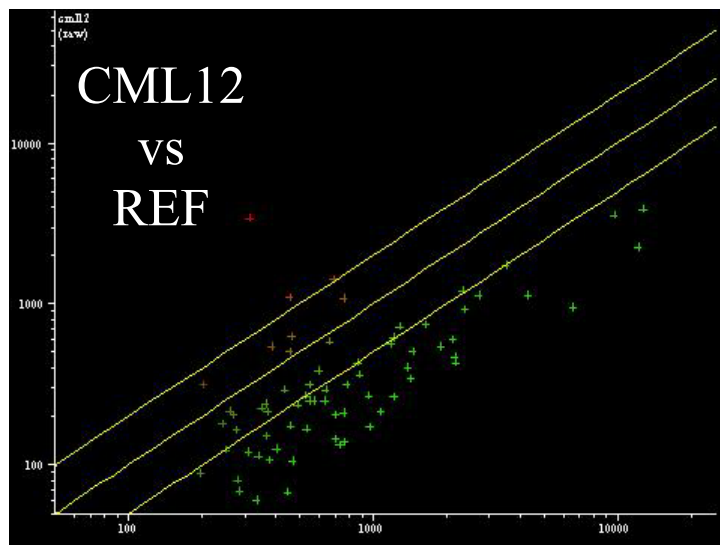
Figure 27: Scatter plot of 58 risk group indicator genes in CML 12. These genes were found to be expressed at least 3-fold higher in high-risk samples. In this figure, CML 12 is on the y-axis and the reference is on the x-axis.

Another confirmation result comes from other array experiments. For example, CML 2 was hybridized against CML 4 and 8. The scatter plot distributions of these 58 genes are given in Figure 28 and 29. In Figure 28, CML 2 (high-risk) is on the y-axis and the CML 8 (low-risk) is on the x-axis. Almost all genes are above the cutoff line. However, in Figure 29, CML 4 (high-risk) is on the y-axis and the CML 2 (high-risk) is on the x-axis. Since both samples are high-risk, all genes are distributed around the cutoff line.
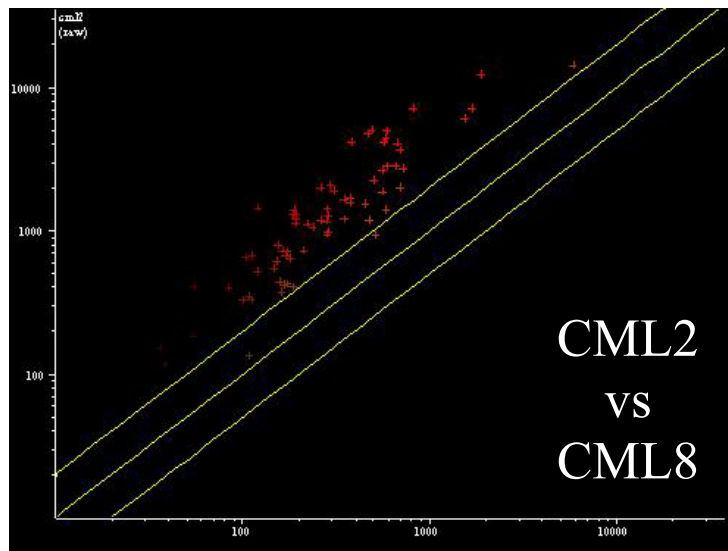
Figure 28: Scatter plot of CML 2 versus CML 8. CML 2 is on the y-axis and the CML 8 is on the x-axis.
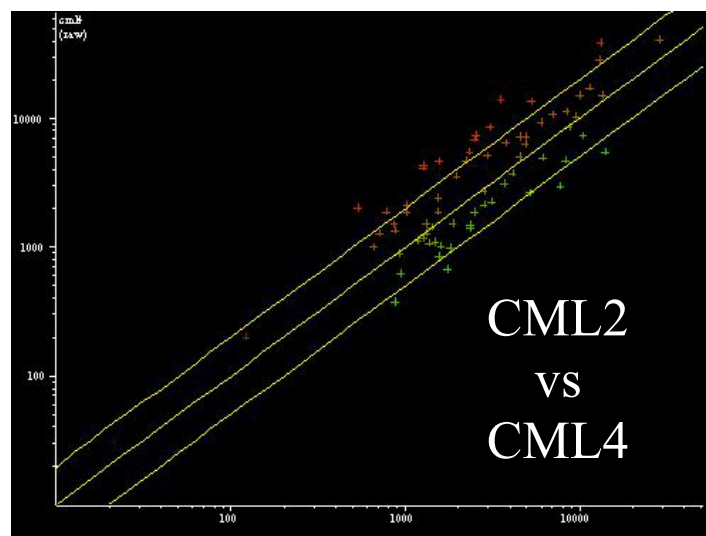
Figure 29: Scatter plot of CML 2 versus CML 4. CML 4 is on the y-axis and the CML 2 is on the x-axis.

3.3 Semi-quantitative RT-PCR analysis

Determination of optimal cycle of a gene for semi-quantitative PCR

To determine the total PCR cycle numbers that will define the logarithmic phase of amplification (optimum cycle not saturated for the amplified DNA fragment), an initial study was performed at 15 through 30 cycles for each primer pair. 80% of PCR product from each cycle was loaded on 2% agarose gel and determined cycle number was used for amplification of the gene of interest in further studies. Figure 30 shows the results obtained from *GAPDH*, *IFITM1*, *UCP2* and *CCNH* genes. The optimal cycle number is 22 for *CCNH,* 21 for *UCP2* and *GAPDH,* and 18 for *IFITM1*.
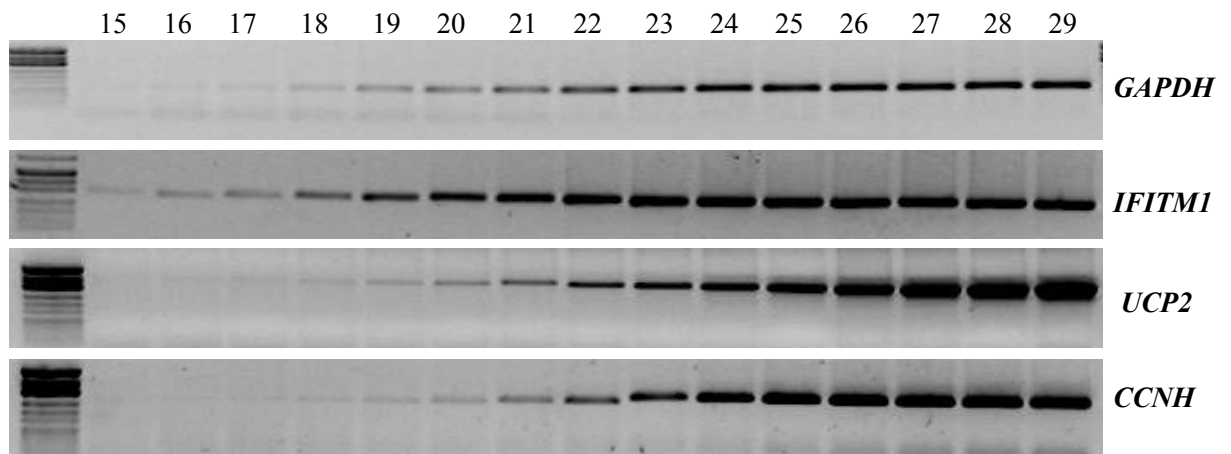
Figure 30: Cycle optimization of *GAPDH, IFITM1, UCP2* and *CCNH* genes. Amplification of each gene was performed by using the primer pairs given in Table 1. 80% of PCR product from each cycle was loaded on 2% agarose gel. $\varnothing$X174 DNA/*Hin*fI Marker, 10 in first lane and cycles 15-29 in the following lanes.

Semi-quantitative RT-PCR results of seven genes in high, low & intermediate risk CML, and other hematological malignancies

To examine relative transcript levels in the remaining patient samples, RT-PCR analysis of seven genes were performed: *IFITM1* (interferon induced transmembrane protein 1)*, CXCL3* (chemokine (C-X-C motif) ligand 3)*, CCNH* (cyclin H)*, RAB1A* (member RAS oncogene family)*, PRKAR2B* (protein kinase, cAMP-dependent, regulatory, type II beta)*, PTGS1* (prostaglandin-endoperoxide synthase 1)*, UCP2* (uncoupling protein 2).

Higher expression of *CXCL3* (9/9), *RAB1A* (7/9), *CCNH* (7/9), *PRKAR2B* (6/9) and *PTGS1* (4/9), and lower expression of *IFITM1* (9/9) and *UCP2* (6/9) was seen in the high-risk patients compared to the low-risk patients (Figure 31A). *PRKAR2B* expression was not detected in any one of the low-risk samples, and *PTGS1* was minimally expressed in only three low-risk samples (Figure 31B). With respect to the intermediate-risk group, gene expression profiles follow the pattern of low-risk samples, except for *UCP2*, which resembles that of the high-risk patients (Figure 31C).

Since *PRKAR2B* expression was observed only in the high-risk CML patients, and similarly *PTGS1* expression appeared to be specific to high-risk CML patients, we analyzed the expression of these transcripts in fourteen patients with other hematological malignancies (Figure 31D). Neither *PRKAR2B* nor *PTGS1* is expressed in any one of these samples, suggesting that expression of these is not a general feature of hematological malignancies.
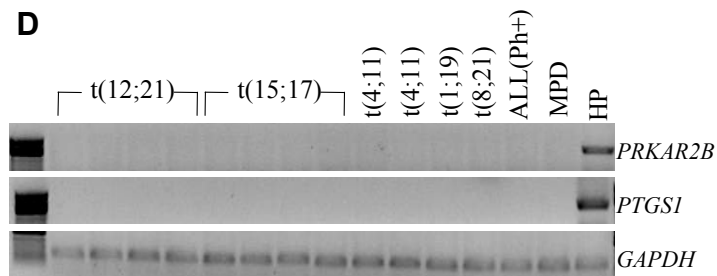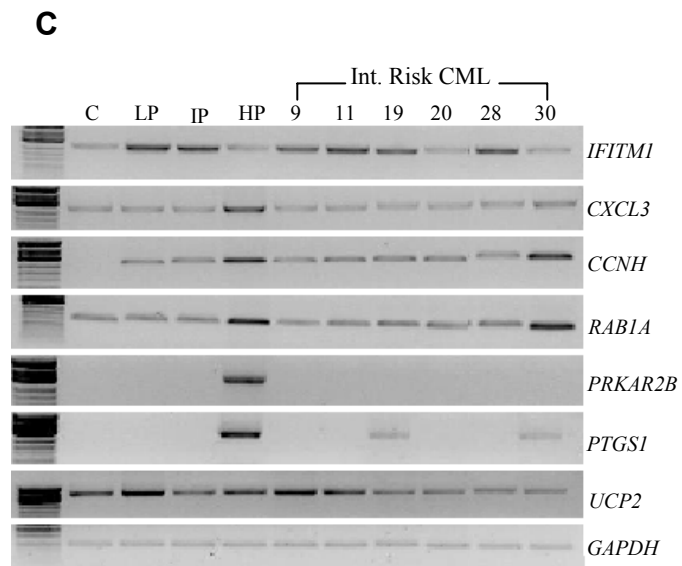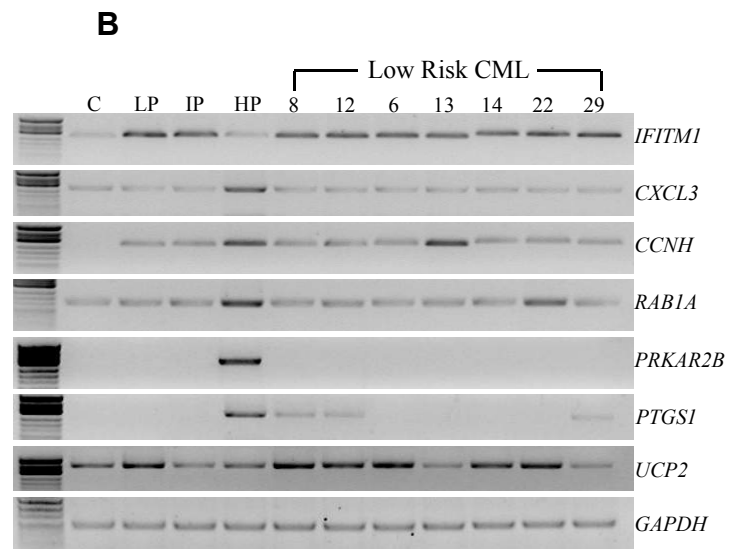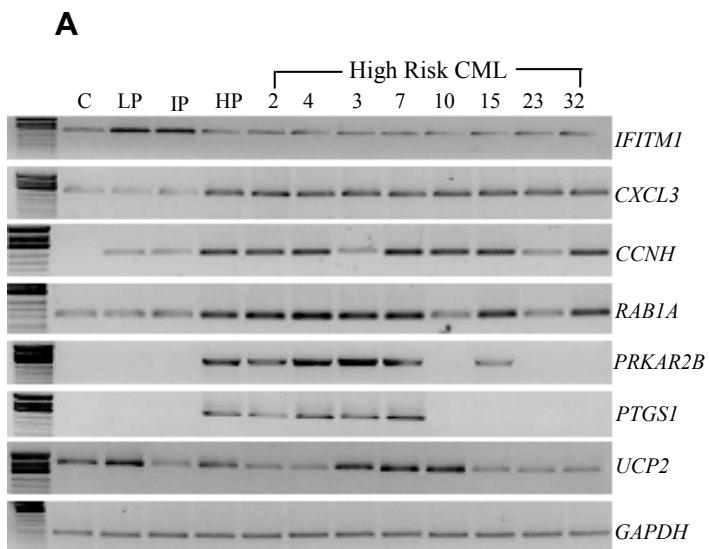
Figure 31: Semi-quantitative RT-PCR results of seven genes in high, low & intermediate risk CML, and other hematological malignancies. **(A)** High-risk CML; ∅X174 DNA/*Hin*fI Marker, 10 in first lane; C: pool of four healthy controls; LP: pool of all low-risk samples; IP: pool of all intermediate-risk samples; HP: pool of all high-risk samples; high-risk CML samples 2, 4, 3, 7, 10, 15, 23, 32 in the following lanes consecutively. **(B)** Low-risk CML; Marker; C; LP; IP; HP; low-risk CML samples 8, 12, 6, 13, 14, 22, 29 in the following lanes consecutively. **(C)** Intermediate-risk CML; Marker; C; LP; IP; HP; intermediate-risk CML samples 9, 11, 19, 20, 28, 30 in the following lanes consecutively. **(D)** Other hematological malignancies; Marker; t(12;21) ALL in lanes 2 to 5; t(15:17) AMLM3 in lanes 6 to 9; t(4;11) infant ALL in lanes 10 and 11; t(1;19) B cell ALL in lane 12; t(8;21) AML in lane 13; t(9;22) ALL in lane 14; myeloproliferative disease in lane 15; pool of all high-risk samples in lane 16.

3.4 Real-time RT-PCR analysis

The relative transcript level of *IFITM1* was determined in forty-nine CML patients by real-time RT-PCR analysis. The results showed that the relative transcript levels were significantly different between the high-risk (ranged between 0.034-0.67; n=11), and low-risk (1.0-6.06; n=34) groups (p=9.7976 x 10^{-11}). The intermediate-risk group (1,62-7,38; n=4) was similar to the low-risk group. The comparison of the relative *IFITM1* expressions in the high and low-risk samples is shown in Figure 32.
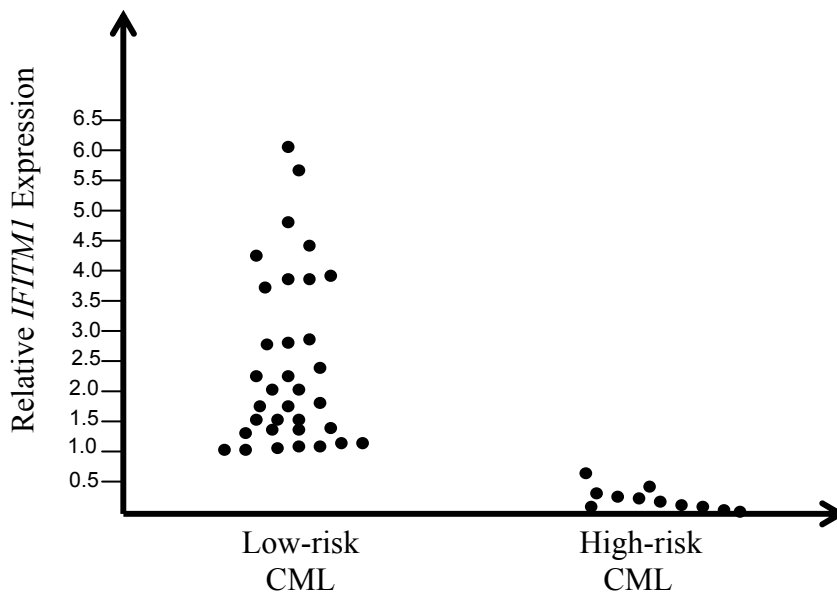
Figure 32: Relative expression of *IFITM1* by real-time PCR in low- and high-risk CML. Levels of gene transcripts between high and low-risk CML were compared and found to be highly significant (p=9.7976 x 10^{-11}).

3.5 Statistical analysis of data

Mann-Whitney U test

Levels of gene transcripts between high and low-risk CML were compared using Mann-Whitney U test (SPSS software version 10.0). P values <0.05 were considered statistically significant. Quantitation of relative band intensities was performed using Multi-Analyst software version 1.1 (Bio-Rad Laboratories). Each product was normalized against *GAPDH*.

Expression levels of all genes except for *PTGS1* are significantly different between the high and low-risk groups (*IFITM1* and *CXCL3* p=0.001; *CCNH* p=0.012; *RAB1A* p=0.01, *PRKAR2B* p=0.016; *UCP2* p=0.04; and *PTGS1* p=0.315).

3.8 Kaplan-Meier analysis for correlation of survival with gene expression

The goal of Kaplan-Meier analysis was to estimate a survival curve from a sample. Vertical axis represents estimated probability of survival for a hypothetical cohort. Horizontal axis represents the time in months. P values <0.05 were considered statistically significant. Quantitation of relative band intensities was performed using Multi-Analyst software version 1.1 (Bio-Rad Laboratories). Each product was normalized against *GAPDH*.

Kaplan-Meier analysis was performed in all patients who have follow-up data for at least 26.5 months (n=24). Kaplan-Meier plot of overall patient survival indicates that low-risk assignment based on NPS does not significantly correlate with increased survival (Figure 33A). However, when patient survival was plotted according to *IFITM1* expression below or above the cutoff value of 2.0 (calculated as median of intensity ratio of *IFITM1* and *GAPDH* amplicons), the low-risk patients demonstrate higher levels of *IFITM1* expression compared to the high-risk patients. This finding significantly correlates with survival (p=0.01; Figure 33B). Similarly, lower *CXCL3* expression correlates with survival (p=0.059; cutoff value 1.0; Figure 33C).

A
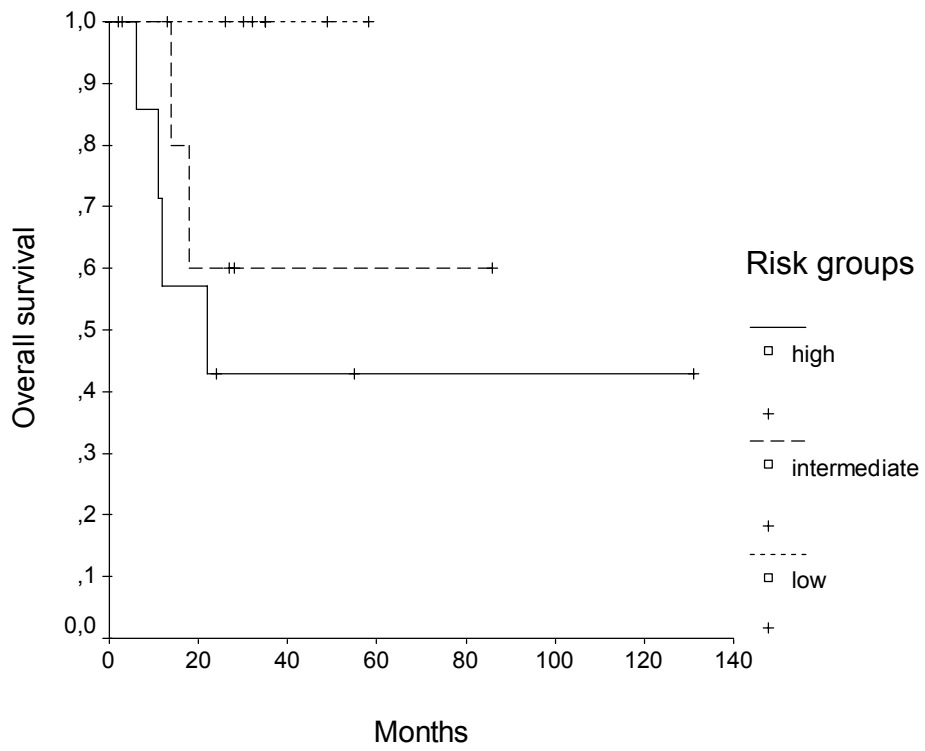
B
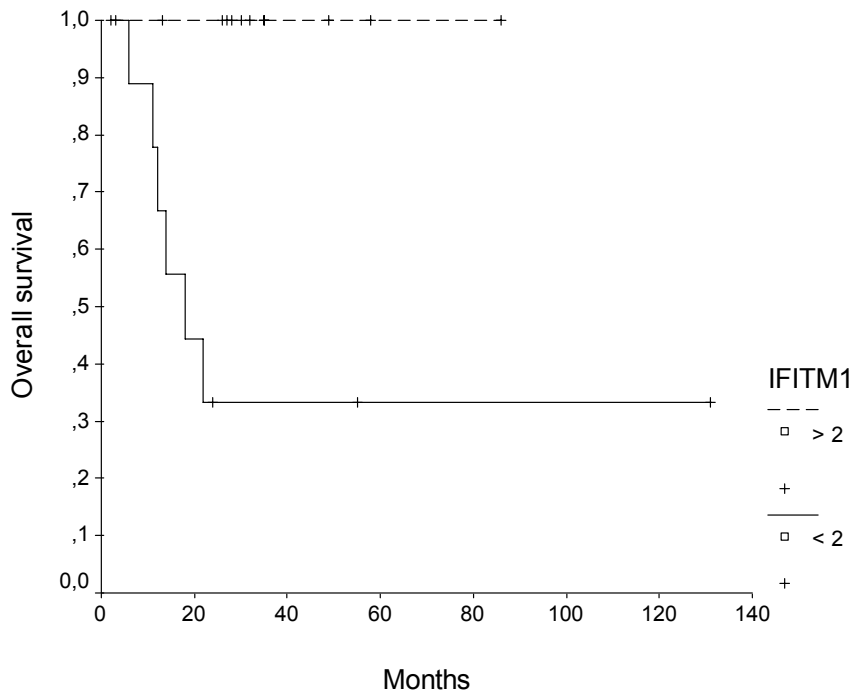
C
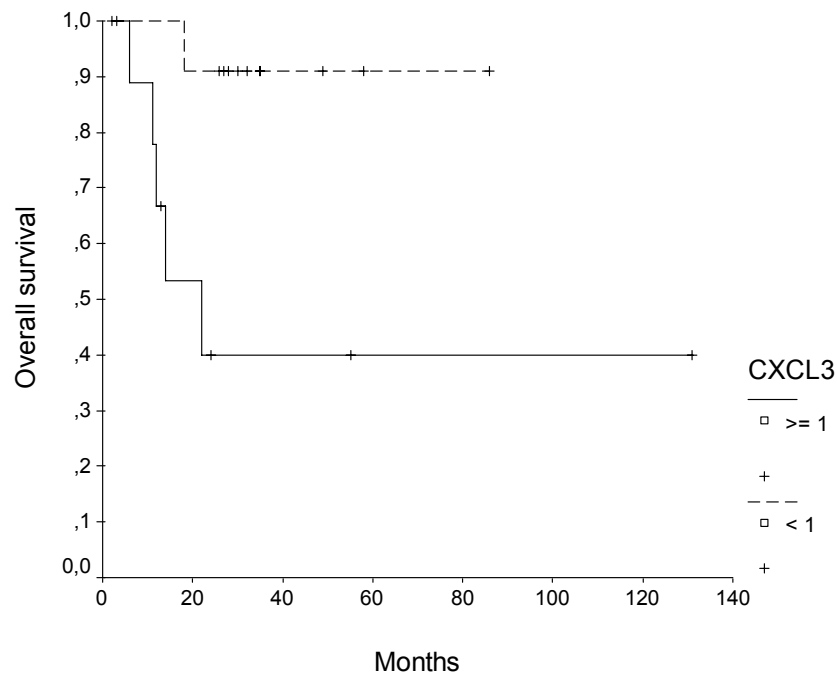
Figure 33: Kaplan-Meier analysis. (A) Kaplan-Meier plot of overall survival of patients according to NPS score (p=0.10). Survival according to (B) *IFITM1* (p=0.01), and (C) *CXCL3* (p=0.059) expression.

3.9 Detection of Gly382Asp *MYH* mutation by *Bgl* II digestion

G1145A point mutation is a missense mutation that leads to G to A substitution at nucleotide 1145 in exon 13 of *MYH* and results in Gly to Asp amino acid substitution at codon 382. The mutation occurs within the recognition site of the restriction enzyme *Bgl* II. Thus, digestion of the *MYH* exon 13 PCR product provides a simple, cost-effective assay for identifying patients who had the mutation. *Bgl* II has no recognition site in the wild type *MYH* exon 13 DNA sequence. The PCR amplified fragment is 242 bp. The G1145A mutation introduces a *Bgl* II site. In a heterozygous individual, the expected fragment sizes are 242 bp, 158 bp and 84 bp, and in a homozygous mutant individual, the expected fragment sizes are, 158 bp and 84 bp.

Amplified *MYH* exon 13 PCR products were incubated with *Bgl* II in the recommended buffer at 37°C for 2 hours. To make sure enzyme is working, a plasmid DNA (pBlueScripSK, 6715 bp) having *Bgl* II recognition sites was also digested. The expected size fragments are 4000 bp, 2200 bp, 515 bp (Figure 34). After digestion, the cut and uncut DNA samples were analyzed on 2% agarose gel. No mutation was observed in any of the patient samples (n=185) and controls (n=124).
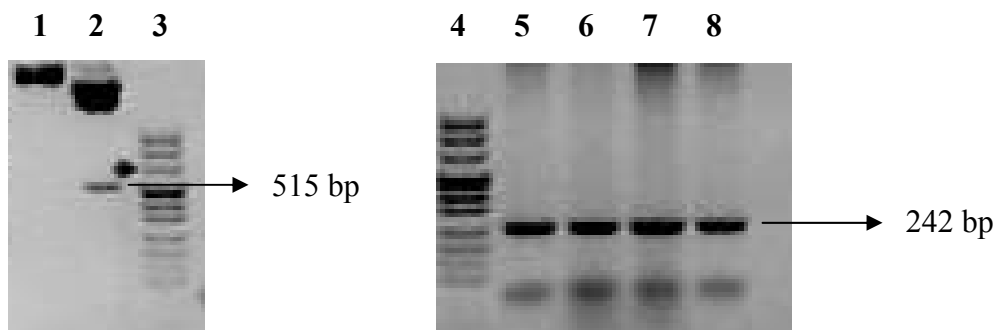
Figure 34: *Bgl* II digestion profile in detection of Gly382Asp. PCR reactions are electrophoresed in 2% agarose. Lane 1: Undigested plasmid pBlueScripSK, Lane 2: Digested plasmid pBlueScripSK, Lane 3: pUC Mix Marker, 8, Lane 4: pUC Mix Marker, 8, Lane 5-8: Digested AML samples, 184, 185, 190, 197.

3.10 Detection of Tyr165Cys *MYH* mutation by ARMS

A494G point mutation is a missense mutation that leads to A to G substitution at nucleotide 494 in exon 7 of *MYH*. As a result, Tyr is substituted to Cys amino acid at codon 165. The size of the PCR fragment produced is 147 bp. Upon the presence or absence of the bands, genotyping was performed in all patient samples and controls by ARMS. The wild-type, heterozygous and homozygous mutant individuals are expected to have A/A, A/G and G/G alleles, respectively. No mutation was present in any of the patient samples (n=184) and controls (n=124), except for that one patient diagnosed with AML/M3. The DNA code for this patient is 02-91. Tyr165Cys mutation in the heterozygous state was present in the sample obtained at the time of initial diagnosis (Figure 35). Further sampling, at remission,

and the analysis of parental DNA, showed only the normal allele. Therefore, the mutation was considered to be specific for the leukemic blasts.
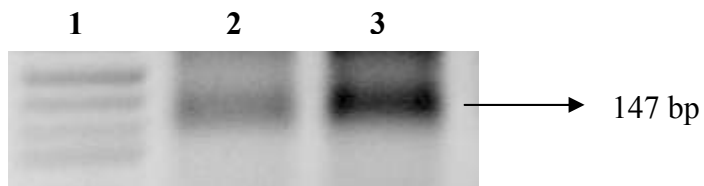
Figure 35: **Detection of Tyr165Cys *MYH* mutation by ARMS.** PCR reactions are electrophoresed in 2% agarose. Lane 1: pUC Mix Marker, 8, Lane 2: PCR of 02-91 with 165N primer, specific to normal allele, Lane 3: PCR of 02-91 with 165M primer, specific to mutant allele.

CHAPTER 4. DISCUSSION

We have studied the gene expression profiles in different risk groups of CML patients at the time of initial diagnosis by using cDNA microarrays and quantitative RT-PCR technique to constitute an initial attempt to identify candidate CML risk group indicator genes. Seven genes, *IFITM1, CXCL3, CCNH, RAB1A, PRKAR2B, PTGS1,* and *UCP2* were chosen from the initial cDNA microarray data and studied. These genes were recovered multiple times on different spots of the arrays and also gave high fluorescent intensity. Expression level of all genes, except *PTGS1,* was significantly different between the high and low-risk groups (*IFITM1* and *CXCL3* p=0.001; *CCNH* p=0.012; *RAB1A* p=0.01, *PRKAR2B* p=0.016; *UCP2* p=0.04; and *PTGS1* p=0.315). Although NPS failed to correlate with patient survival, higher *IFITM1* or lower *CXCL3* expression correlated with improved survival (p=0.01, p=0.059 respectively).

Although gene expression profiles associated with CML have been reported (Ohmine *et al.,* 2001; Li *et al*., 2002), to the best of our knowledge, this is the first study in which gene transcript levels at initial diagnosis are correlated with clinical parameters and survival. Our results show that the expression levels of *IFITM1* and *CXCL3* were significantly different between the high- and low-risk groups. Higher *IFITM1* (p=0.01) and lower *CXCL3* (p=0.059) expressions correlated with improved survival. *IFITM1*, a component of a multimeric complex involved in the trunsduction of antiproliferative and cell adhesion signals implicated in the control of cell growth (Deblandre *et al.,* 1995), was suggested to play a role in the antiproliferative activity of interferons (Gutterman, 1994). The sensitivity to inhibition of cell growth induced by interferons was found to correlate with the expression of this gene in various cell lines (Knight *et al.,* 1985; Evans *et al.,* 1990; Evans *et al.,* 1993). Furthermore, culture of human RSa cells with interferon-α

resulted in increased resistance of the cells to cell killing by X-rays, and increased levels of *IFITM1* mRNA (Kita *et al*., 2003).

These observations support our finding that high-risk CML samples which are expected to have a high proliferative capacity, show decreased *IFITM1* expression levels. In another study, *IFIT2* (interferon induced protein with tetratricopeptide repeats-2) was found to be the most highly expressed gene during chronic phase CML (Ohmine *et al*., 2001). These results suggests that higher expression of interferon induced genes in CML patients may serve as an indicator of interferon-α sensitivity, which in turn may be used as a molecular marker to predict response to interferon-α treatment. *IFITM1* could be a molecular marker to identify patients in different CML risk groups based on the observations that this gene has a role in the antiproliferative activity of interferons, and low-risk CML patients respond better to interferon-alfa treatment. In an independent study, *IFITM1* was found to be increased in multiple myeloma (MM) versus normal twin plasma cells (PCs) (Munshi *et al*., 2004). Genetic heterogeneity between individuals confounds the comparison of gene profiling of MM cells versus normal PCs. To overcome this barrier, the gene expression profile of CD138(+) MM cells from a patient bone marrow (BM) sample with CD138(+) PCs from a genetically identical twin BM sample was compared using microarray profiling. Despite cell survival pathway genes, oncogenes, and transcription factors, *IFITM1* was found to be 32.4 fold increased in MM cells versus normal twin PCs.

With respect to the function of *CXCL3*, the available information implicates its involvement in inflammation and on endothelial cell functions in an autocrine fashion (Haskill et al. 1990; Tekamp-Olson et al. 1990). In addition, it may be constitutively overexpressed in transformed human cells (Anisowicz et al. 1987). High *CXCL3* expression in high-risk CML is supported by these observations.

IFITM1 and *CXCL3* expression levels do not appear to be directly correlated with the blast counts of the patients based on the real-time and semi-quantitative RT-PCR results. For example, the highest blast counts in low-risk patients were observed for CML 8 (blasts 5%), CML14 (5%), and CML 22 (8%), but the *IFITM1* expression levels were high and *CXCL3* levels were low (Fig.31B). Among the

high-risk patients, although some had blast counts similar to low-risk patients, as exemplified by CML 4 (8%), CML 2 (7%), CML 10 (4%), the *IFITM1* and *CXCL3* expression levels did not resemble the low-risk patients (Fig.31A).

Although we are not able to present the quantitative expression of *HLADRB1* in all patients, our microarray results of higher expression in the two low-risk patients agree with previous findings in solid tumors and acute myeloid leukemia, where increased *HLADRB1* expression was associated with less metastasis or improved survival (Moos *et al.,* 2002; Bernsen *et al.,* 2003).

We also compared the chromosomal localization of high-risk indicator genes with the recurrent chromosome aberrations reported for CML (Mitelman Database of Chromosome Aberrations in Cancer. (http://cgap.nci.nih.gov/Chromosomes/Mitel_Search#MARK). Most interestingly, *PTGS1* at 9q33.2 lies very close to the t(9;22) breakpoint. Furthermore, *PRKAR2B* at 7q22.3 and *CCNH* at 5q14.3 map near unbalanced 7q22 and 5q13 translocations respectively. Finally, *RAB1A* at 2p14 maps close to balanced translocations at 2p13.

We have shown that candidate CML risk group indicator genes can be identified using gene expression profiling, and this profiling may lead to the development of a gene expression based classification system for CML which appears to be highly correlated with the clinical scoring at the time of initial diagnosis. Furthermore, a gene expression based classification system for CML may more accurately predict disease outcome.

In the second part of this study, we screened for the *MYH* Tyr165Cys and Gly382Asp variants using genomic DNA in childhood AML and ALL. Neither mutation was present in any of the patient samples and controls, except for one patient diagnosed with AML/M3. Tyr165Cys mutation in the heterozygous state was present in the sample obtained at the time of initial diagnosis. Further sampling, at remission, and the analysis of parental DNA, showed only the normal allele. Therefore, the mutation was considered to be specific for the leukemic blasts. It may be interesting to screen the whole *MYH* gene for mutations in hematological malignancies in future, especially if increased transversions of G:C to T:A proved to

be present in leukemic blasts. Based on these results, an association between childhood leukemias and the *MYH* missense variants Tyr165Cys and Gly382Asp was not observed. Also, these variants appear to be absent -if not at a very low frequency- in the Turkish population, contrary to British population.

CHAPTER 5. FUTURE PERSPECTIVES

The CML risk group indicator genes identified in this study may lead to the development of a gene expression based classification system for CML which appears to be highly correlated with the clinical scoring at the time of initial diagnosis. Since the candidate genes identified in our study are novel and have not been previously analyzed in different populations, further studies are needed to increase the impact of our initial screening.

Monitoring *IFITM1* expression levels at given time intervals in patients who are treated with interferon-α might help to determine the validity of utilizing *IFITM1* expression profiling as a potentially important molecular marker in CML.

Structural and numerical chromosome aberrations and mutations have long been recognized in cancer development. But recent observations indicate epigenetic alterations as equally important players in carcinogenesis including hematological malignancies. It is plausible that the genes we identified to be differentially expressed in different CML risk groups are subject to epigenetic changes. DNA methylation plays an important role in the establishment and maintenance of epigenetic changes in the human genome. The major epigenetic modification of human genomic DNA is the methylation of cytosine residues located within the dinucleotide CpG. Several genes have been shown to be independently hypermethylated in hematological malignancies (Esteller, 2003). Based on these considerations, two types of studies could be performed in our patient samples. First, mutations can be analyzed in genes associated with the addition of methyl residues to the DNA molecule or genes that constitute the transcription repression complex. In addition, the methylation pattern of the genes that we identified as differentially expressed in different CML risk groups can be analyzed by employing new genome

analysis technologies such as sodium bisulfite modification of DNA followed by methylation-specific PCR (MSP) (Gutierrez *et al*., 2003).

In the second part of this study, *MYH* Tyr165Cys and Gly382Asp variants were screened in childhood leukemias. It may be interesting to screen the whole *MYH* gene for mutations in hematological malignancies in the future, especially if increased transversions of G:C to T:A proved to be present in leukemic blasts.

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APPENDICES

a. **The patient information form**

Bilkent Üniversitesi/Ankara Üniversitesi/The Cleveland Clinic Foundation
Moleküler Biyoloji ve Genetik Bölümü
KML Chip Çalışması Formu

(İstenilen örnek EDTA'lı tüplerde 30-50 ml kan)

Hastanın Adı ve Soyadı: _____

Yaşı: _____ Cinsiyeti: ___ Mesleği: _____ Dosya numarası: _____

Adresi: _____

_____

Telefon no (ev) : _____ Telefon no (cep) : _____

Telefon no (iş) : _____ Faks no : _____

Doktorun Adı ve Soyadı: _____

Bölümü ve Üniversitesi : _____

Telefon no: _____

Hasta tanısı: _____ Tanı zamanı: _____

Tanıyı koyan doktor: _____

Diğer hastalıklar: _____

Hastanın kan değerleri ve dalak büyüklüğü:

		Tanı sırasında	Şimdi
a. Lökosit sayısı	:	_____	_____
b. blast sayısı (%)	:	_____	_____
c. eosinofil sayısı (%)	:	_____	_____
d. bazofil sayısı (%)	:	_____	_____
e. platellet sayısı (10^9/L):		_____	_____

f. dalak büyüklüğü (kosta sınırından itibaren kaç cm): _____

Hastanın risk gurubu: Yüksek: [ ] Düşük: [ ]

Hastanın tedavi durumu (kemoterapi, interferon, vs): _____

Tedaviye başlama tarihi ve protokolü: _____

Hücre kodu: _____ cDNA kodu: _____

RNA kodu: _____ DNA kodu: _____

b. The informed consent form

BİLGİLENDİRİLMİŞ ONAY FORMU*

Kendimde KML hastalığı olup olmadığının anlaşılması için FISH ve/veya RT-PCR ile t (9:22) incelemesi işleminin yapılması gerektiği ve işlem hakkında bilgiler bana aktarıldı. Bunun yanında Bilkent ve Ankara Üniversiteleri ile The Cleveland Clinic Foundation arasında başlatılan KML hastalığının tedaviye cevabı ile ilgili araştırma projesi çerçevesinde interferon sinyal ileti yolu incelemesi yapılacağı belirtildi.

Bu bilgiler ışığında KML hastalığı genetik incelemesi işleminin yapılmasını onaylıyorum.

Hasta   Doktor

(İsim Soyad, İmza)   (İsim Soyad, İmza)

Şahit

(İsim Soyad, İmza)

*Ref. T.C. Sağlık Bakanlığı, Ana Çocuk Sağlığı ve Aile Planlaması Genel Müdürlüğü, Genetik Hastalıklar Tanı Merkezleri Yönetmeliği, (1998)den uyarlanmıştır.

c. The raw fluorescent data obtained from arrays of CML patients and control

Genbank	CML4 Normalized	Raw	Control	CML2 Normalized	Raw	Control	CML8 Normalized	Raw	Control	CML12 Normalized	Raw	Control
AA279147	48,2	18579,0	385,5	52,4	29958,0	571,2	14,3	6010,5	419,1	11,2	3407,5	305,6
AA181500	21,7	14130,0	650,0	17,1	8959,5	522,8	1,1	1318,0	1213,3	2,5	1092,5	445,9
W47101	21,4	7552,0	352,4	10,6	5275,0	497,9	1,7	975,0	567,5	1,6	313,5	197,3
AA181500	17,2	25020,5	1454,0	10,2	14596,0	1436,6	1,6	3891,5	2367,9	2,1	1416,0	678,2
R06438	15,2	7536,0	494,4	5,3	3741,0	710,5	0,9	658,0	696,4	1,4	541,5	376,0
N70773	12,4	14783,5	1191,7	6,1	7664,5	1261,2	0,6	140,0	224,6	0,5	250,0	540,3
W47101	10,3	4143,5	403,1	8,6	7619,5	890,3	2,1	2105,0	1007,7	1,4	623,5	453,6
T74192	7,4	7259,0	977,9	4,7	5870,0	1258,7	0,8	695,5	885,9	1,1	500,0	447,8
N22684	6,4	5035,0	790,3	2,2	2166,0	1006,1	0,4	619,5	1516,6	0,7	224,0	343,9
AA935273	6,1	5201,5	847,3	3,2	3951,0	1221,1	0,6	915,0	1559,2	0,4	249,0	619,3
AA454668	5,2	10662,5	2033,9	4,4	10610,0	2398,2	1,3	1090,0	841,3	1,4	1076,0	742,4
AA482286	4,8	4195,5	877,5	3,6	3032,0	843,9	0,6	556,5	1008,7	0,7	237,5	357,3
W86182	4,3	17047,0	3959,5	4,5	12235,0	2694,3	0,4	872,0	2432,0	0,5	564,0	1160,0
AA707321	4,3	10896,0	2531,1	4,1	8820,5	2157,3	0,3	533,5	1823,4	0,5	427,0	850,2
N73222	3,7	8356,5	2249,4	3,6	9350,5	2565,8	1,2	4327,5	3614,2	0,9	582,5	649,5
W86182	3,7	11067,5	2985,0	4,0	10459,0	2634,2	0,3	697,0	2298,7	0,5	234,5	479,4
AI026973	3,5	1871,0	541,1	2,3	1565,0	679,2	0,3	237,5	770,6	0,8	179,0	237,1
AA707321	3,5	8635,5	2500,3	3,6	10834,0	2998,8	0,3	1260,5	3852,4	0,5	614,0	1193,1
AA463544	3,5	6361,5	1842,3	3,2	5478,0	1724,4	0,4	92,0	248,2	0,4	359,0	861,7
R36587	3,4	3267,0	962,5	5,4	4364,5	807,7	0,4	434,5	977,6	0,9	217,0	253,9
AA913804	3,4	2124,0	629,5	4,5	2967,0	665,0	0,8	814,0	1031,8	0,2	68,0	277,8
W42723	3,3	2188,0	659,7	4,4	4528,0	1035,4	0,6	786,5	1407,3	0,5	268,5	518,7
N78902	2,8	1288,5	454,4	2,1	1235,5	597,1	0,4	355,5	904,9	0,4	151,5	358,3
N24824	2,7	1262,0	462,4	1,3	1439,5	1089,7	0,4	626,0	1672,5	0,3	205,0	688,7
AA456869	2,5	3671,5	1442,0	2,2	3080,5	1371,6	0,3	624,5	2090,1	0,7	290,0	425,3

AA463544	2,5	2650,5	1074,8	2,7	2376,0	872,7	0,3	242,0	886,4	0,3	112,5	331,9
AA425238	2,4	802,5	332,4	2,4	862,5	359,6	0,7	208,0	301,8	0,8	203,0	263,0
AA453593	2,4	1125,0	466,4	2,4	3955,5	1682,9	0,6	157,5	276,2	0,7	383,0	587,7
N78902	2,3	1376,5	586,7	2,1	1660,5	794,0	0,3	346,0	1007,2	0,5	88,0	193,5
AA287107	2,2	1482,0	659,7	1,0	1074,0	1095,0	0,2	242,5	1490,5	0,3	107,0	368,3
H54020	2,2	8235,0	3815,8	2,1	10460,5	5001,7	0,3	1745,0	5595,6	0,6	716,5	1263,5
R92806	2,1	12908,0	6059,5	3,2	21844,5	6850,7	0,6	1254,5	2001,4	0,4	1120,0	2652,5
AA127116	2,0	43569,5	22128,9	1,6	20094,0	12597,0	0,3	9773,0	36803,0	0,3	3842,5	12429,1
T71308	1,9	1211,5	624,4	3,1	1936,5	625,9	0,4	429,0	960,6	0,5	124,0	243,8
R20770	1,9	2113,5	1090,2	1,9	2705,0	1422,9	0,5	760,0	1457,9	0,4	173,0	449,7
AA044390	1,9	2995,5	1582,9	1,9	2693,0	1423,4	0,1	214,0	1710,6	0,4	248,0	557,0
N75017	1,9	1969,5	1050,9	2,4	2498,0	1047,6	0,5	515,5	1065,9	0,6	211,5	361,6
AA489232	1,9	1081,5	579,3	2,6	2232,0	860,5	0,6	480,5	831,2	0,6	163,0	270,6
R27581	1,9	2843,0	1524,7	1,9	3415,0	1814,8	0,4	331,5	832,7	0,6	315,0	540,3
N75017	1,8	2771,5	1545,8	2,7	3876,0	1446,9	0,4	145,0	361,5	0,5	290,0	627,9
NM_001959	1,7	34761,0	20892,7	0,7	20583,0	31510,2	0,2	7145,5	37928,1	0,2	2234,0	11920,9
AA019511	1,7	8226,5	4977,8	3,6	13258,0	3689,7	0,5	4288,5	7928,3	0,5	1211,0	2274,1
AA460930	1,6	7640,0	4689,3	2,1	18974,0	9042,7	0,4	240,5	600,6	0,5	1730,5	3407,8
AA416785	1,6	18323,5	11311,1	2,0	28808,0	14478,8	0,2	1603,0	9208,3	0,4	3537,0	9495,0
H50229	1,6	1184,0	734,4	0,8	1422,5	1723,9	0,2	408,0	2293,2	0,2	144,0	690,7
AW075605	1,6	16156,0	10196,3	1,2	18155,5	14624,9	0,2	2987,0	16249,7	0,3	1118,0	4210,6
NM_003750	1,6	3983,0	2515,2	1,5	4688,5	3102,4	0,4	1070,5	2866,2	0,5	737,0	1608,8
R93829	1,5	7956,5	5247,0	2,1	11108,0	5231,8	0,3	471,0	1719,6	0,4	922,5	2323,0
N47717	1,5	4258,5	2933,1	0,5	1864,0	3442,4	0,2	424,5	2816,6	0,2	171,5	951,7
AA454146	1,4	1293,0	923,2	1,2	1296,0	1120,0	0,3	210,0	815,2	0,3	166,0	522,5
AA448814	1,4	3155,0	2264,8	1,7	3470,5	2032,2	0,4	1664,5	3708,5	0,4	311,5	766,3
AA412053	1,3	5279,0	3957,2	1,9	7820,0	4044,4	0,2	742,0	3837,3	0,3	601,5	2081,6
AA476508	1,3	630,5	486,4	1,5	1056,5	706,6	0,4	245,0	586,1	0,3	80,5	273,0
AA465603	1,3	2288,0	1787,6	1,2	2575,5	2171,0	0,2	544,0	2410,0	0,2	105,5	456,9
AA454146	1,2	1333,0	1104,5	1,3	2047,0	1576,8	0,2	259,0	1324,6	0,4	120,5	304,1

Genbank	Normalized	Raw	Control	Normalized	Raw	Control	Normalized	Raw	Control	Normalized	Raw	Control
N69689	1,2	3556,5	2969,0	1,9	7457,0	3948,7	0,2	1020,0	4961,4	0,4	505,5	1424,0
AA460981	1,2	1267,5	1064,0	1,0	1198,0	1183,5	0,2	238,0	1115,0	0,3	125,5	393,2
N67039	1,2	1241,5	1058,3	1,2	2041,5	1711,7	0,1	227,0	1875,5	0,2	131,5	717,0
AA099383	1,1	1466,5	1371,9	1,5	2979,5	1972,6	0,2	529,0	2292,2	0,3	210,0	743,8
N59851	1,0	1221,5	1171,8	0,6	631,5	1118,0	0,1	265,0	1824,9	0,2	67,0	435,9
AA446819	1,0	2453,0	2391,4	2,7	16207,5	6064,0	0,3	139,5	433,2	0,3	537,0	1855,5
R52654	1,0	4148,0	4338,1	1,5	8272,0	5510,4	0,1	547,0	4887,7	0,2	467,5	2118,9
AA598526	0,9	2811,5	3096,2	1,0	2613,0	2649,4	0,2	630,5	3045,2	0,3	268,5	944,0
NM_001530	0,9	2603,5	2881,8	0,9	2221,5	2349,4	0,2	398,0	2400,0	0,3	400,5	1351,2
AA424824	0,8	5666,5	6986,7	0,6	5258,5	8373,3	0,1	1409,5	10130,3	0,2	432,0	2140,0
R52654	0,8	3158,0	4001,1	1,1	5837,5	5181,5	0,1	225,5	2007,9	0,2	339,0	1394,3
AA406332	0,7	2767,0	4059,3	0,8	2837,5	3387,7	0,1	462,5	4260,5	0,2	265,5	1193,6
AW004895	0,7	10525,5	15511,7	0,5	8996,0	17218,6	0,0	506,0	15336,8	0,1	941,0	6451,2
AA001745	0,6	2015,5	3310,0	0,7	2199,0	3121,4	0,2	443,0	2250,1	0,2	213,5	1055,6
N93924	0,6	1311,5	2281,9	0,7	1240,5	1804,0	0,1	190,0	2856,2	0,2	137,5	748,6
AA598526	0,6	792,5	1379,3	0,8	745,5	971,9	0,1	176,0	1241,8	0,2	60,0	327,1

	CML4			CML2			CML8			CML12		
Genbank	Normalized	Raw	Control	Normalized	Raw	Control	Normalized	Raw	Control	Normalized	Raw	Control
AA630549	2,8	3779,0	1369,6	3,1	3509,5	1150,3	70,3	63612,5	905,4	22,9	11893,0	520,2
AA630549	2,5	2658,5	1057,2	2,8	1938,0	694,8	57,2	34017,0	594,6	17,0	6273,5	369,3
T63324	1,2	477,5	391,2	1,6	825,0	528,7	23,0	11408,0	495,8	6,2	2150,5	348,2
AA634006	5,9	14252,5	2425,6	4,4	9133,0	2069,4	22,0	9339,5	425,1	23,3	21937,0	941,2
H08749	1,7	2393,0	1387,3	5,9	9095,0	1531,4	20,3	33854,5	1664,5	24,8	12853,5	518,2
T63324	1,5	761,5	505,2	1,6	604,5	369,9	18,1	4546,5	251,2	5,5	818,5	148,5
AA634006	3,7	13258,5	3538,7	3,4	2296,0	669,4	17,8	15211,0	854,8	14,4	14105,0	978,0
T63324	1,4	1105,5	777,2	1,3	945,0	714,4	16,2	2737,5	169,5	4,3	1600,0	370,2
AA436459	1,7	1438,5	840,5	2,6	3149,5	1224,0	11,7	15128,5	1290,0	10,1	3181,0	315,6
H61243	2,8	4042,5	1434,6	2,5	4803,5	1937,9	11,4	26987,0	2373,4	14,4	9471,5	658,6

AI871056	0,5	633,0	1302,3	1,1	2323,5	2080,6	9,5	13972,5	1474,0	3,7	3788,5	1031,2
AA931043	0,5	1042,0	1931,8	1,2	4772,5	3835,3	8,4	51895,0	6148,0	18,9	24027,5	1274,5
T83159	2,2	1687,0	750,4	2,3	1281,0	547,8	8,3	2785,0	334,9	10,7	2279,0	213,6
T58146	1,5	716,0	470,4	2,4	1046,5	444,7	7,4	1884,0	255,2	7,6	1494,5	197,8
AA931043	0,5	740,5	1601,1	1,1	1518,5	1433,7	5,2	4816,0	921,0	23,1	5608,0	242,8
AA599177	0,9	3216,5	3579,1	0,8	2360,5	3013,9	5,2	10055,0	1941,7	4,5	3390,5	751,0
AA933862	0,7	390,0	593,6	0,9	655,5	768,6	5,0	2616,5	520,9	3,8	1310,5	341,5
AI253411	1,3	2002,5	1494,5	1,6	1790,0	1145,4	4,9	772,0	156,4	4,8	2648,5	556,1
AA620426	1,3	993,0	747,5	1,4	949,5	685,6	4,2	556,5	133,4	4,4	1186,0	270,6
T63324	0,6	560,0	977,3	0,4	485,5	1123,9	4,2	2601,5	626,2	2,2	513,5	234,7
AA419251	0,6	2048,5	3469,7	0,6	4533,0	7438,5	4,1	29003,0	7074,5	6,0	16324,0	2721,9
N69335	1,1	952,5	862,7	1,2	620,5	532,1	3,9	1316,5	336,9	4,2	1055,5	254,3
N69335	1,0	1267,5	1293,2	1,2	1078,5	920,6	3,9	3261,5	843,8	5,2	2482,0	479,0
AA480071	0,3	272,5	834,2	0,3	307,5	881,0	3,2	974,5	308,3	1,1	664,0	596,8
NM_002168	0,3	2555,5	8814,7	0,4	4178,5	11283,1	2,2	20898,0	9643,5	3,9	11829,0	2997,8
AA458965	0,2	181,0	1065,1	0,4	706,5	1771,3	2,1	1466,5	695,4	8,2	4685,5	570,0
T47442	0,6	398,0	719,6	0,6	685,5	1201,6	2,0	2318,0	1142,6	1,7	905,0	526,4
AA444009	0,3	519,5	1764,2	0,5	808,0	1638,4	1,7	2498,0	1488,5	2,1	1419,5	682,5
AA630328	0,2	815,0	4908,9	0,3	1969,0	6954,8	1,5	3703,5	2507,2	3,8	5422,5	1429,7
AI675889	0,4	119,0	310,2	0,4	175,5	421,2	1,3	312,0	243,2	2,8	802,5	285,9
AA838691	0,2	103,5	503,5	0,2	314,0	1296,8	1,0	1282,0	1224,3	1,4	660,5	472,3
AI208702	0,3	571,5	2185,6	0,3	414,0	1510,9	0,8	699,5	846,8	1,2	619,5	500,5

RAPID COMMUNICATION

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Analysis of *MYH* Tyr165Cys and Gly382Asp variants in childhood leukemias

Introduction

DNA-repair gene mutations have been suspected as being a predisposing factor in the development of leukemia (Horwitz 1997). Our group identified one of the first examples of a DNA-repair gene mutation to be causally linked to childhood hematological malignancies and neurofibromatosis type I, which involves a homozygous germ-line mutation in the mismatch repair (MMR) gene *MLH1* (Ricciardone et al. 1999; Wang et al. 1999). Subsequently, homozygous inactivation of *MSH2*, another MMR gene, was also found to be associated with early onset leukemia (Whiteside et al. 2002). It is well known that heterozygous germ-line mutations in the MMR pathway genes *MLH1*, *MSH2*, *PMS2*, *PMS1*, and *MSH6* lead to hereditary non-polyposis colorectal cancer (HNPCC) (Peltomaki 2001). Thus, tumorigenesis through an ''MMR deficiency pathway'' appears to be associated with two different disease phenotypes which are dependent on the status of the germ-line mutation: (a) HNPCC when the mutation is present on only one allele (heterozygous), and (b) hematological malignancies when the mutation(s) is present on both alleles (homozygous or compound heterozygous).

Base excision repair (BER) is another important DNA-repair pathway and plays a significant role in the repair of mutations generated by reactive oxygen species during aerobic metabolism. BER was not linked with any human genetic disorder until recently, when a British family in which three siblings affected by multiple colorectal adenomas and carcinoma was shown to be compound heterozygous for *MYH* missense variants Tyr165Cys (Y165C) and Gly382Asp (G382D) (Al-Tassan et al. 2002). MYH is a homologue of *E. coli* mutY, and the mutations mentioned above affect residues that are conserved (Tyr82 and Gly253). Tyrosine 82 is predicted to function in mismatch specificity and is located in the pseudo-helix-hairpin-helix (Guan et al. 1998). Adenine glycosylase activity assays of the Tyr82Cys and Gly253Asp mutant proteins with 8-oxoG:A and G:A substrates show that their rate for adenine removal at 37°C is reduced by approximately 98% (Tyr82Cys) and 86% (Gly253Asp) (Al-Tassan et al. 2002). Furthermore, bi-allelic germ-line mutations in *MYH* were identified in seven unrelated patients with colorectal adenomas (six with colorectal cancer) (Jones et al. 2002). Interestingly, the missense variations Tyr165Cys and Gly382Asp, which significantly reduce the adenine glycosylase activity of MYH protein, were each identified once in a normal control group of 100 British individuals with no history of colorectal adenoma or carcinoma (Al-Tassan et al. 2002). Since a connection between DNA-repair gene mutations and the path to hematological malignancy is now well established, and individuals who carry heterozygous *MYH* missense mutations Tyr165Cys and Gly382Asp have been documented in a control group (Al-Tassan et al. 2002), we investigated the association between these two *MYH* missense mutations and childhood leukemia risk.

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Results and discussion

The study population included 185 cases of childhood leukemias subdivided into two groups: acute myeloid leukemia (AML; $n = 45$) and acute lymphoblastic leukemia (ALL; $n = 140$) diagnosed at Istanbul University between 1998 and 2002. Detailed clinical data are available for all patients. The French–American–British

Cooperative Study Group criteria were used for histological subgroup classification (Cheson et al. 1990). Randomly selected Bilkent University students, with no history of hematological malignancies or any other type of cancer ($n = 124$), were genotyped in order for us to assess the status of the *MYH* mutations in apparently healthy Turkish individuals. Informed consent was obtained from all the students.

We screened for the *MYH* Tyr165Cys and Gly382-Asp variants using genomic DNA as described (Al-Tassan et al. 2002). Neither mutation was present in any of the samples, except for that of one patient diagnosed with AML/M3. *MYH* Tyr165Cys mutation in the heterozygous state was present in the sample obtained at the time of initial diagnosis. Further sampling at remission, and the analysis of parental DNA, showed only the normal allele. Therefore, the mutation was considered to be specific for the leukemic blasts. It may be interesting to screen the whole *MYH* gene for mutations in hematological malignancies in the future, especially if increased transversions of G:C to T:A proved to be present in leukemic blasts. Based on these results, an association between childhood leukemias and the *MYH* missense variants Tyr165Cys and Gly382Asp was not observed. Also, these variants appear to be absent—if not at a very low frequency—in the Turkish population, contrary to the British population.

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