CYTOGENETICS AND MOLECULAR STUDIES IN CHRONIC MYELOID LEUKEMIA

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ABSTRACT

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This PhD Thesis consists of two parts: cytogenetic studies and molecular studies in chronic myeloid leukemia (CML).

Cytogenetic studies was performed on a total of 256 newly diagnosed Malaysian CML patients from January 1995 to December 1999. The bone marrow aspirate of the CML patients was collected in transport media and sent to the Genetics Laboratory, Hematology Division, Institute for Medical Research, Kuala Lumpur for cytogenetic studies. A density cell count was performed and the cells were cultured with culture media containing colcemid overnight at 37^0C. Harvesting was done the next day. The cells were subjected to hypotonic treatment, fixed with fixative, dropped onto cold wet slides, aged, banded, and stained. Good metaphase spreads was selected and captured using the Cytovision Satellite Capture Station, and karyotyped using the Cytovision Karyotyping Work Station. Interpretation and reporting was done according to the International System for Human Cytogenetic Nomenclature, 1995.

The results of the cytogenetic studies are as follows: Out of the 256 newly diagnosed CML patients, 222 (86.7%) have the Philadelphia (Ph) chromosome and 34 (13.3%) have a normal karyotype. Among the 222 Ph-positive (Ph+) CML patients, 204 (91.9%) have the standard t(9;22) translocation and 18 (8.1%) have variant translocation (9 patients with simple variant translocation and another 9 patients with complex variant translocation). At presentation of the disease, 33 (14.9%) of Ph+ CML patients have additional chromosome abnormalities.

Molecular studies that is *bcr-abl* gene rearrangement studies was performed on a total of 52 CML patients from December 1998 to February 2001. All these 52 patients was found to have the Ph chromosome from cytogenetic studies performed by the

Genetics Laboratory, IMR. For normal control, gene rearrangement studies was done on ten normal individuals.

About 2 ml of peripheral blood of the CML patients and normal control was collected into an EDTA bottle. The white blood cells was isolated using ficoll-paque, and Trizol was used to extract the RNA from the white cells. A one step RT-PCR (reverse transcriptase-polymerase chain reaction) was performed using the following primers and enzymes: Abl3+ and NBl+ primers, *AMV* reverse transcriptase and *Tfl* DNA polymerase. The amplified products was subjected to another round of PCR (nested PCR) with CA3 and B2 primers to detect the *bcr-abl* gene rearrangement, and CA3 and A2 primers as the control. The amplified nested PCR products was subjected to agarose gel electrophoresis, viewed under UV light and photographed.

Out of the 52 Ph+ CML patients studied 19 (36.5%) patients had b2a2 and 33 (63.5%) had b3a2 transcripts. The presence of the b2a2 or/and b3a2 transcripts indicate the presence of the *bcr-abl* gene rearrangement (that is the Ph chromosome at the cytogenetics level). 51 of the CML patients have the standard t(9;22) translocation and one had a variant translocation, t(18; 22). This patient was found to have a b2a2 transcript.

TABLE OF CONTENTS

2.3.1 : Transport media for collection of bone marrow aspirate

2.3.2 : Bone marrow culture media

2.3.3 : Colcemid 5ug/ ml

2.3.4 : Bromodeoxyuridine

2.3.5 : Sodium salt heparin

2.3.6 : Potassium chloride (KCl), 0.075M

2.3.7 : Leishman stain

2.4 : Methodology for cytogenetic studies

2.4.1 : Checking of specimen and recording of patients' particulars

2.4.2 : Cell density calculation (done in biohazard cabinet)

2.4.3 : Setting up culture

2.4.4 : Harvesting (ONC and BONC)

2.4.5 : Slide making

2.4.6 : Banding and staining

2.4.7 : Screening of metaphase spreads

2.4.8 : Capturing of metaphase spreads

2.4.9 : Karyotyping of chromosome spreads

2.4.10 : The Cytovision system

2.4.10A: Cytovision karyotyping workstation

2.4.10B: Brightfield capture

2.4.11C: Cytovision satellite capture station

2.5 : Materials for molecular studies

2.5.1 : Reagents for molecular studies

2.5.2 : Equipment for molecular studies

2.6 : Preparation of reagents

2.6.1 : RT-PCR reaction, Tube M

2.6.2 : Nested PCR reaction, Tubes A and B

2.6.3 : Preparation of Tube A

2.6.4 : Preparation of Tube B

2.7 : Methodology for molecular studies

CHAPTER ONE
INTRODUCTION

4.1 Origin of blood cells

All blood cells, such as granulocytes, neutrophils, eosinophils, monocytes, erythrocytes, macrophages, B and T lymphocytes, platelets and natural killer cells originate from a lymphohemopoietic stem cell in the bone marrow. The lymphohemopoietic stem cell has an extensive self renewal and proliferative potential as well as the capacity to differentiate into the progenitors of all blood cell lineages. Self renewal refers to the ability to produce daughter cells with identical characteristics. The sequential development of progenitor cells and mature cells from stem cells is controlled by circulating or membrane-bound cytokines. The hierarchal model of lymphohemopoiesis is shown in Fig. 1.1. A very small proportion of circulating stem cells can be isolated from blood by special techniques (Quesenberry, 1995).

The level of blood cells is controlled by multiple humoral and cellular factors and adjusts rapidly according to requirement. For example, infection due to microorganisms will result in an immediate release of mature neutrophils, and an increase production of granulocytes and monocytes, until the infectious agents are cleared. Hemorrhage or acute hemolysis will result in a rapid release of marrow reticulocytes, and an increase in red cell production until the red cell numbers return to normal.

1.2 Hemopoietic stem cell disorders

The hemopoietic stem cell disorders are classified into two principal pathogenetic disorders, aplasia and clonal hemopathies (Table 1.1). Aplasia is due to stem cell failure

while clonal hemopathies is due to an injury to a single cell within the hemopoietic stem cell pool (Lichtman, 1995a).

4.1.1 Aplasia

Aplastic pancytopenia, also known as aplastic anemia, is a result of aplasia or suppression of hemopoietic stem cells, or of the progenitors of all cell lineages. This leads to a deficiency of each blood cell type. The disease is heterogenous, and several different mechanisms of stem cell suppression may be involved.

4.1.2 Clonal hemopoiesis

Clonal hemopoiesis includes the preleukemias and myeloproliferative disorders (MPDs). The various types of preleukemias and MPDs are shown in Table 1.1.

Chronic myeloproliferative disorders are clonal hematopoietic stem cell diseases due to excessive production and overaccumulation of erythrocytes, granulocytes, and platelets in some combination in the bone marrow, peripheral blood, and body tissues. The chronic MPDs include chronic myeloid leukemia (CML), polycythemia vera, primary thrombocythemia, idiopathic myelofibrosis, chronic monocytic leukemia and chronic neutrophilic leukemia. Acute myeloid leukemia (AML) develops as a terminal event in each of these clonal stem cell diseases, ranging from 75% in patients with CML to 15% in patients with primary thrombocythemia and idiopathic myelofibrosis (Lichtman, 1995a)

1.3 Leukemia

The incidence of leukemia in the world is about 1 per 100,000 per year (Cartwright, 1992). Malignant neoplasms (including leukemia) accounts for about 10.5% of medically certified death in Malaysia in 1996 (Annual Report 1996). Leukemia arises when an immature blood cell in the bone marrow (progenitor cell) develops uncontrollably and suppresses the production of healthy blood cells. The unregulated proliferating cells usually replace normal marrow, interfere with normal marrow function, may invade other organs and eventually cause death if untreated. Chronic leukemia was first described by European physicians in 1845. These patients have a chronic disease of about 1-2 years and at autopsies large spleens and elevated white cell count was noted. Because of this, the

condition was referred to as *weisses blut* (German for 'white blood'). Later the word 'leukemia' was used for the disease (Greek *leukos*, 'white'; *haima* 'blood). Acute leukemia was described later in 1877 where patients with 'white blood' was noted to die rapidly.

In the 1900s it was established that leukemia can be acute (progressing quickly with many immature blasts) or chronic (progressing slowly with more mature-looking cancer cells). Based on morphological and cytochemical studies, the acute leukemia is divided into two groups: AML and acute lymphoblastic leukemia (ALL). However, today with the combination of morphological, cytogenetics, immuophenotyping, cytochemistry, and molecular studies, the accuracy in the classification and diagnosis of leukemia have increased tremendously. AML and ALL each are further divided into subtypes based on the French American British (FAB) classification. (Bennett et al, 1981; 1985). Chronic leukemia can be divided into those of lymphoid and myeloid origin. Examples of chronic leukemias are chronic lymphocytic leukemia (CLL), hairy cell leukemia, and chronic myelomonocytic leukemia (CMMOL). CML also called chronic granulocytic leukemia (CGL) is best considered a chronic myeloproliferative disorder (MPD).

1.4 Chronic myeloid leukemia (CML)

CML is a chronic MPD arising as a clonal process in a pluripotent hematopoietic stem cell. The disease is characterized by anemia, granulocytosis, basophilia, thrombocytosis, and splenomegaly. The disease has a high propensity to evolve into acute leukemia at the terminal stage.

1.4.1 Epidemiology of CML

CML represents about 20% of all cases of leukemia and the death rate is about 1.5 per 100,000 population per year. The disease is more frequent in men than women. CML tends to be a disease of middle life with a slowly increasing age trend in Western countries. The greatest incidence of CML is in the age range 40-60 years. About 10% of CML cases are found in subjects between 5 and 20 years of age. CML represents about

3% of all childhood leukemias. There is no concordance of the disease between identical twins (Lichtman, 1995b).

4.1.1 Etiology of CML

There is an increase in frequency of CML in people exposed to ionizing radiation. People who were exposed to the atomic bomb radiation at Nagasaki and Hiroshima, patients with ankylosing spondylitis treated with spine irradiation, and women with uterine cervical carcinoma treated with radiation therapy, had an increased frequency of CML (and acute leukemia) compared to the unexposed group. The mean latent period was about 4 years in irradiated spondylitis patients, 9 years in uterine cancer patients, and 11 years in the Japanese survivors of the atomic bombs. The incidence of CML was 2.6 times higher from the Hiroshima blast than from the Nagasaki blast, while the rates of acute leukemia are about equal. Since the type of radiation differed in the two places, it is possible that this may have contributed to the different distributions (Ichimaru et al, 1978; Maloney, 1985).

Chemical leukemogens have not been identified as causative agents of CML. Evidence of CML as an inherited disease is very weak when compared to CLL (Gunz, 1997).

1.4.3 Origin of CML from a stem cell

CML results from the malignant transformation of a single stem cell. The disease is acquired (somatic mutation). The following evidence shows that CML originates from a single stem cell (Lichtman, 1995b):

(a) The involvement of erythropoiesis, neutrophilopoiesis, eosinophilopoiesis, basophilopoiesis, monocytopoiesis, and thrombopoiesis in the chronic phase of CML.

(b) The presence of the Philadelphia (Ph) chromosome in erythroblasts, granulocytes, macrophages, plasma cells and megakaryocytes (Haferlach et al, 1997).

(c) The presence of a single glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme in red cells, neutrophils, eosinophils, basophils, monocytes, and platelets, but not in fibroblasts or other somatic cells in women with CML who are heterozygotes for isoenzymes A and B.

(d) The presence of the Ph translocation only on a structurally anomalous chromosome 9 or 22 of each chromosome pair in every cell analyzed.

(e) The presence of the Ph chromosome on one but not the other cell lineage of patients who are mosaic for sex chromosomes.

(f) Molecular studies show precisely the same breakpoint among cells within a single CML patient.

(g) Combined DNA hybridization-methylation analysis of women with restriction length polymorphisms at the X-linked locus for hypoxanthine phosphoribosyltransferase (HPRT) which enables the distinction of the two copies of HPRT gene in heterozygous females, together with the methylation-sensitive restriction enzyme cleavage patterns which enables the distinction between maternally and paternally derived genes.

4.1.1 Clinical features of CML

The most common presentation is anemia, loss of energy, fatigue, dyspnoea, anorexia, weight loss and pallor. During the chronic phase of the disease, neutropenia is absent and thrombocytopenia uncommon. An increasing number of patients are detected when asymptomatic, for example, when blood tests are performed, blood is donated or as part of a regular physical evaluation.

The most common physical findings are pallor and splenomegaly. The spleen is palpable in 80-90% of patients. Absence of splenomegaly is usually associated with a leucocyte count of less than 100×10^9 /l. In patients with very high leucocyte counts, usually exceeding 500×10^9 /l, the retinal veins are dilated and the ophthalmoscopic appearances resemble those of Waldenstrom's macroglobulinemia

CML can be diagnosed from the results of the blood counts and examination of peripheral blood films. There is a complete spectrum of granulocytic forms, ranging from blast cells to mature polymorphonuclear cells. The differential leucocyte count is characterised by two peaks, myelocytes and mature neutrophils, with lesser numbers of metamyelocytes. The total leukocyte count is elevated at the time of diagnosis and is over 25×10^9 /l, and half of the patients have total white counts over 100×10^9 / l. The total leukocyte count rises progressively in untreated patients (Spiers, 1992).

The abnormal laboratory findings in CML include a low neutrophil alkaline phosphatase activity, elevated serum levels of vitamin B_{12} and its binding protein transcobalamin 1, and hyperuricaemia. However, non of these findings are specific tfor the disease.

CML is characterized by two discrete phases: a chronic phase of about 3 years duration and a terminal acute phase which lasts from one-half to one year. At the acute

phase, about 75% of CML patients develop AML and 25% ALL (Lichtman, 1995a). In 50-75% of patients, there is an intervening accelerated phase associated with increased myelopoiesis and resistance, or inability to tolerate therapy (Dreazen et el, 1988).

1.4.5 Therapy of CML

Busulfan in doses of 4 to 6 mg per day, orally can be used until the white cell count falls to about 30 x 10^9 /l. The effect of the drug persists for days to weeks, and a further decrease may occur towards or to normal levels. Chronic use of busulfan has been associated with a syndrome that stimulates adrenal insufficiency, manifested by skin pigmentation, weakness, fever, and diarrhoea, or with pulmonary fibrosis. Prolonged aplasia of the marrow can occur with busulfan.

Hydroxyurea is now preferred to busulfan for the treatment of CML. It is less toxic, may sustain the chronic phase of the disease for a longer time, and may permit greater success with marrow transplantation therapy. The major side effect of hydroxyurea is that it causes suppression of hemopoiesis which is reversible. The median survival of CML patients treated with hydroxyurea is about 5 years, and is longer than for patients treated with busulfan, which is about 3.75 years.

Interferon-α can result in a normalization of blood counts in about three-quarters of CML patients at the chronic phase of the disease. About one-third of the patients treated intensively for several months with interferon-α have a decrease in Philadelphia-positive (Ph+) cells, and 15% have less than 5% Ph+ve cells. The greater the decrement in Ph+ cells, the longer the survival of the CML patient. Patients with at least a 50% reduction of Ph+ cells have a 5-year survival of about 90%. However the disadvantage of interferon-α is that it has to be administered intramuscularly, and there are early side effects (e.g. fever, fatique, anorexia, nausea, etc) as well as later side effects (e.g. apathy, thrombocytopenia, renal or cardiac dysfunction, etc).

Patients in the chronic phase of CML and who are less than 55 years of age and who have an identical twin, a histocompatible sibling, or access to a histocompatible donor, can be treated with bone marrow transplantation (BMT). BMT can eradicate the Ph+ cells, and has led to apparent cure of some CML patients.

4.1.1 Cytogenetic findings in CML

Chromosome abnormalities in neoplasia are acquired. The abnormalities are

restricted to the tumor tissue, highly nonrandom and clonal in nature. Two main mitotic events, abnormal segregation and chromosome breakages can affect the chromosomes in neoplastic cells. Abnormal segregation (e.g. nondisjunction, anaphase lag) results in changes in chromosome numbers that range from monosomy and trisomy to haploidy and polyploidy. Chromosome breakages caused by exogenous factors (e.g. ionizing radiation, viruses, chemical mutagenic agents) and endogenous factors (e.g. enzyme systems comprising DNA replication, transcription, and recombination) results in deletions, duplications, inversions, translocations, insertions , and amplifications.

Chromosome aberrations in neoplasia are classified into primary and secondary abnormalities. Primary abnormalities are strongly nonrandom, correlated with a specific malignancy, may be the only cytogenetic abnormality, and may play a role in the initiation of the malignancy at the early stages. Secondary abnormalities are also nonrandom, but less disease specific, and are postulated to be later events contributing to the process of tumour progression.

In the clinical setting, cytogenetic analysis is an invaluable tool in the diagnosis, prognosis, and management of hematological malignancies. It also aids in the differential diagnosis between solid tumour types with common features.

In 1960 Nowell and Hungerford discovered a deletion in chromosome 22 in patients with CML. The 'shortened' chromosome 22 was called the Philadelphia or Ph chromosome in honour of the city it was first identified. This was a major discovery as the Ph chromosome was the first consistent abnormality identified in a malignancy.

Cytogenetic studies reveal that about 90-95% of CML patients have the Ph chromosome whereby one chromosome 22 appears to be 'shortened'. More than 90% of the Ph chromosome is due to the standard t(9;22)(q34.1;q11.2) reciprocal translocation (Fig. 1.2). The remaining 5-10% Ph+ CML patients have variant translocations which may be complex involving chromosomes 9, 22 with one or more other chromosomes; or simple involving chromosome 22 with a chromosome other than 9 (Fig. 1.3). High- resolution banding studies have demonstrated simple and complex variant translocation to cryptically involve translocation of 9q34 to 22q11. In rare cases, a reciprocal translocation with a chromosome other than 9 to chromosome 22 is larger than usual, and the post

translocation shortening of the long arms of 22 is inapparent. Since the 22q- is not evident by microscopic examination, it has been referred to as a masked Ph chromosome or masked translocation (Fig. 1.3).

Molecular studies have shown the involvement of chromosome 9 in variant translocation. There is no difference in the clinical and hematological picture between patients with the standard and variant translocation. The role of the Ph chromosome in the etiology of CML has not been fully established. Some studies suggest that it may appear after the initial leukemogenic event. Patients with CML who developed the Ph chromosome during the course of the disease has been observed.

At diagnosis of Ph+ CML, 10-20% of the patients have additional cytogenetic abnormalities; mainly an additional Ph chromosome, trisomy 8 and in males the loss of the Y chromosome (Thompson & White, 1992). The presence of these additional abnormalities at diagnosis has been shown to be associated with a poor prognosis.

A change in the karyotype of a patient with only the Ph chromosome is an ominous sign. Secondary chromosome aberrations occur in 75-80% of CML patients when disease progression occurs. The major (70%) routes of cytogenetic evolution in CML accelerated phase and blast crisis are listed in Table 1.2. Gains of chromosome 8, an extra Ph chromosome, and isochromosome for the long arm of chromosome 17 [i(17q)], are the most common solitary changes. Combinations of these changes along with gain of chromosome 19 usually occur later in karyotype evolution. More than 50% of minor route cases (30%) include –Y, -7, -17, +17, +21 or t(3;21)(q26;q22). Near haploid leukemia stemlines have also been reported (Block 1999). In addition to the common secondary changes, disease progression is also associated with marker chromosomes. M-FISH studies has enabled the identification of these marker chromosomes (Gribble et al, 2000).

There is a correlation between the type of secondary chromosome aberration with the phenotype of the blast crisis. Hyperdiploidy, trisomy 8, 19, 21, and i(17q) are associated with myeloid blast crisis. An extra Ph is seen in both myeloid and lymphoid blast crisis. Chromosome loss is seldom seen (-7 is the most common) compared to chromosome gain in blast crisis. These changes can precede the blastic phase by 2-4 months. Followup cytogenetic studies would be a valuable tool to monitor the progression of the disease, especially an impending blast crisis (Le Beau & Rowley, 1995; Block,1999).

About 5-10% of CML patients do not have the Ph chromosome. These Ph negative (Ph-) patients differ from Ph+ CML patients in a number of ways (older, male, shorter survival, lower platelet and white blood count). The common chromosome aberrations found in Ph- CML patients are trisomy 8 (20%), monosomy 7 (20%), i(17q) (10%), and structural rearrangements of chromosome 3 (>3%) (Block, 1999). Most Ph-

patients have some form of myelodysplasia such as CMMOL or refractory anemia with excess blasts. Molecular studies has shown that about 1-2% of Ph- CML patients have *bcr-abl* gene rearrangement. However, although rare, the existence of 'true CML' in Ph- CML patients without *bcr-abl* gene rearrangement have also been found (Costello et al, 1995).

The Ph chromosome is found not only in CML but in 15-30% of adults with ALL, 3-5% of children with ALL, and 1-2% of adults with AML (Ribeiro et al, 1987). The presence of the Ph chromosome in acute leukemia carries a poor prognosis.

1.4.7 Molecular biology of CML

The c-*abl* proto-oncogene is mapped to 9q34.1 (Fig. 1.4) which is the site of breakage in the t(9;22) translocation. C-*abl* is the normal cellular homologue of the transforming gene of *Abelson* murine leukemia virus, a virus that causes pre-B cell leukemia in mice. It is 230kb in length, consists of at least 11 exons and is orientated with its 5'end towards the centromere). Two alternative first exons exist (exons a1 and b1). Exon a1 is 19kb proximal to exon 2, while exon b1 is > 200 kb proximal to exon 2. This genomic configuration results in the transcription of two types of mRNA: the 6-kb mRNA which consists of exons a1 through 11, and the 7-kb mRNA, which begins with exon b1, skips the 200-kb distance to exon 2, and joins to exon 2 through 11 (Shtivelman et al, 1986). Only one protein product of molecular mass of 145 kD has been identified, called $p145^{abl}$. The normal protein $p145^{abl}$ shows weak tyrosine kinase activity. The close association of $p145^{abl}$ with other tyrosine phosphokinase enzymes indicates that it may be involved in the control of normal cell growth.

The breakpoint cluster region (*bcr*) gene on chromosome 22q11.21 is 130kb in length (Fig.1.5), and expresses a 4.5 and 6.7-kb mRNA. The translation product of the *bcr* gene is a 160-kD protein, $p160^{bcr}$ which can oligomerizes, autophosphorylates and transphosphorylates several protein substrates. The first exon sequences of the *bcr* gene potentiate the tyrosine kinase of the *abl* when they fuse as a result of the translocation. The central portion of the *bcr* has homology to *dbl*, a gene involved in the the control of cell division after the S-phase of the cell cycle. The C-terminus of the *bcr* has a guanosine triphosphatase (GTP)-activating protein for p21, a member of the *ras* family of GTP-binding protiens (Diekmann et al, 1991; Hall, 1992) . $p160^{bcr}$ interacts with the abl SH2 domain. A reciprocal hybrid gene, *abl-bcr*, is formed on chromosome 9q+ when *bcr-abl* fuses on chromosome 22q-. The *abl-bcr* fusion gene actively transcribes in most patients with CML (Melo et al, 1993).

The breakpoints on chromosome 22 in patients with Ph+ CML are clustered within a 5.8-kb DNA segment designated as the breakpoint cluster region (bcr) in the central portion of the *bcr* gene. This region has also been referred to as the major breakpoint cluster region (M-bcr). In contrast, the breakpoints on chromosome 9 are more widely dispersed, most frequently occurring in the first intron of the *abl* gene. Eighty nine percent of CML patients have breakpoint within the 175-kb first intron between exons b1 and a1 of the c *abl* gene (Jiang et al, 1990).

As a result of the t(9;22) translocation, all 3' *bcr*-gene exons distal to the point of breakage within M-bcr are physically removed and relocated to chromosome 9. Proximal 5' *bcr*-gene exons including *bcr* exons 1 and 2, with or without exon 3 remain on chromosome 22. The c-*abl* exons 2 (a2) through 11 are transposed to chromosome 22 and exons a1 and b1 may be included as well. During the process of mRNA formation the splice acceptor site associated with c-*abl* exon a2 can skip splice donor sites in c-*abl* exons a1 and b1 to fuse with splice donor sites of the juxtaposed *bcr*. Two potential junctions are encoded depending on whether *bcr* exon 2 (b2) or *bcr* exon 3 (b3) is joined to c-*abl* a2. These juntions are designated as either b2a2 or b3a2 depending on the absence or presence of *bcr* exon b3. The presence of b2a2 would suggest the existence of the omission of *bcr* exon 3 due to the mechansim of alternative splicing. They differ in size by 75bp at the mRNA level and 25 amino acids at the protein level. Both proteins have elevated tyrosine kinase activity, a molecular weight of 210 kD ($p210^{bcr\text{-}abl.}$) [Fig. 1.6], and can phosphorylate tyrosine residues on cellular proteins similar to the action of the *v-abl* protein product.

The $p210^{bcr\text{-}abl}$ differs from the normal $p145^{abl}$ by its end terminal being substituted by *bcr* sequences. The $p210^{bcr\text{-}abl}$ can transform hematopoietic stem cells *in vitro* (McLaughlin et al, 1987) and this could be one of the many steps leading to leukemogenesis. The $p210^{bcr\text{-}abl}$ can also prolong the survival of progenitors and granulocytes by inhibiting the genetically determined normal program of cell death. The effect may result in the expansion of the cells derived from the leukemic clone (Bedi et al, 1994).

In some patients, coexpression of b2a2 and b3a2 junctions has been reported (Lee et al, 1989). Variations in the breakpoints have been reported such as rearrangement outside the M-bcr (Selleri et al, 1987). In a few cases of CML, where there has been no evidence of elongation of chromosome 9, molecular probes have shown that *abl* is still translocated to chromosome 22.

Pajor et al, 2000 reported one patient with features uncharacteristic of CML having the Ph chromsosome [t(9; 22)] detected by cytogenetic studies at presentation of the disease, but was negative for *bcr-abl* gene rearrangement. Followup studies on this patient eventually showed the presence of b3a2 transcript. Although rare, Ph negative CML with the *bcr-abl* gene located on chromosome 9 (instead of the usual 22) has also been reported (Macera et al 1998).

The c-*sis* proto-oncogene on chromosome 22 (q12.3 to 13.1) is the normal cellular homologue of the transforming gene of simian sarcoma virus which causes sarcomas in non-human primates. Unlike the c-*abl* proto-oncogene, the breakage in the t(9;22) translocation does not occur within the c-*sis* protooncogene although c-*sis* proto-oncogene is translated from chromosomes 22 to 9. The c-*sis* gene is closely related or identical to the gene encoding the β-chain of platelet derived factor, a factor which stimulates collagen synthesis by fibroblasts. C-*sis* can be expressed in the accelerated phase but not in chronic phase CML. The transcript when expressed is normal in size, (4kb) [Romero et al, 1986]. Activation of *cis* is not related to progression of the disease. Genes such as N-*ras*, K-*ras*, and *myc* could play a role in the onset or progression of the disease.

4.1.1 Molecular basis of Ph chromosome-positive acute leukemia

The Ph chromosome in acute leukemia is not only associated with breaks within the M-bcr (Ph+ bcr+). Breakpoint can also be at 5'upstream from M-bcr but generally within the first intron of the bcr gene of chromosome 22. This region has been designated the minor breakpoint cluster region (m-bcr). As a result of rearrangement within m-bcr, the *bcr-abl* fusion gene give rise to a 7.0kb mRNA owing to joining of the first exon (e1) of the *bcr* gene to the second exon a2 of the *c-abl* gene (Blennerhassett et al, 1998). The translocation product is a protein of 190 kD ($p190^{bcr\text{-}abl}$)with enhanced tyrosine kinase activity. $p190^{bcr\text{-}abl}$ has a more potent effect than $p210^{bcr\text{-}abl}$ (Kelliher et al, 1991). This group of Ph+ bcr- (rearrangement of m-bcr) acute leukemia represents *de novo* acute leukemia. Fig. 1.6 shows the e1a2 fusion gene, 7.0kb mRNA and $p190^{bcr\text{-}ab\ l}$ in Ph+ bcr- ALL.

Ph+ acute leukemia may be biphenotypic (lymphoid and myeloid lineage) or myeloid–lymphoid hybrid.

a) Myelogenous

About 1-2% of adult AML patients have the Ph chromosome. There are two varieties of Ph+ AML; that is CML presenting in myeloid blast crisis (Ph+ bcr+ AML), and *de novo* AML (Ph+ bcr- AML) (Lichtman 1995b).

Arguments in favour of CML presenting in myeloid blast crisis (Ph+ bcr+ AML) are as follows:

i) Blast crisis may occur within days after diagnosis of Ph+ CML.

ii) Some cases presented with additional cytogenetic abnormalities comparable to CML blast crisis.

iii) The presence of marked hepatosplenomegaly.

iv) The presence of normal platelet counts and intermittent increases of basophils.

v) Prolonged period of weakness, weight loss and appearance of some clinical features of CML.

vi) Poor prognosis like CML in myeloid blast crisis.

vii) At remission, the disease resembles CML at chronic phase.

viii) Rearrangement at the M-bcr on chromosome 22, production of *bcr-abl* fusion gene and translation of $p210^{bcr\text{-}abl}$ protein product, similar to CML. resulting in the production of $p210^{bcr\text{-}abl}$.

Arguments in favour of de novo AML (Ph+ bcr- AML) are as follows:

i) Additional chromosomal abnormalities are different from those seen in CML myeloid blast crisis.

ii) The Ph chromosome is associated with breaks outside the M-bcr (at the m-bcr) and a translation product of $p190^{bcr\text{-}abl}$.

b) Lymphocytic

About 3-5% of childhood ALL and 15-30% of adult ALL have the Ph chromosome. Again, the presence of the Ph chromosome in ALL carries a poor prognosis. Molecular studies show that there are two groups of Ph+ ALL: CML presenting in lymphoid blast crisis (Ph+ bcr+ ALL) and *de novo* ALL (Ph+ bcr- ALL). Some adults and few children with ALL have rearrangement within the M-bcr and production of $p210^{bcr\text{-}abl}$ (Ph+ bcr+ ALL or lymphoid blast crisis of CML). At remission, patients may revert to CML, and are Ph+.

Some adults and virtually all children with ALL have rearrangement outside the M-bcr, that is at the m-bcr and translation of a $p190^{bcr\text{-}abl}$ protein product (Ph+ bcr- ALL or de novo ALL). At remission, there is appearance of normal hemopoiesis (Lichtman, 1995b)

1.5 Cytogenetic studies

1.5.1 Introduction and history of cytogenetic studies

Cytogenetic studies of human cells can be divided into three phases. The first phase involved the development of methods to obtain metaphase spreads. Initial culture techniques used skin fibroblasts, and in 1956 Tijo and Levan identified the correct number of chromosomes in man. Four years later phytohemagglutinin (PHA) was discovered to stimulate T-lymphocytes in the peripheral blood to undergo mitosis. This transforms cytogenetic analysis from a research tool to a diagnostic tool. In 1960, the Ph chromosome was found to be the first consistent chromosome abnormality to be associated with a malignancy.

Cytogenetic studies from 1956 to 1969 were performed using Giemsa stain resulting in the chromosomes staining uniformly along their length. Identification of chromosomes was difficult especially when chromosomes were of similar size and shape. A nomenclature to group chromosomes according to size was accepted at the Denver Conference in 1960, and this system was modified at the Chicago Conference in 1967.

The development of chromosome banding techniques from 1969 to 1971 led to the second phase of cancer cytogenetics. The Paris Conference in 1971 established the convention used for numbering each chromosome and chromosome band. In 1972, the first consistent translocation involving chromosomes 8 and 21 [t(8;21)(q22;q22)] was identified in AML. It was then also discovered that the Ph chromosome is not due to a deletion but a reciprocal translocation involving chromosomes 9 and 22 [t(9;22)(q34;q11)] (Rowley, 1993). Chromosome translocations, deletions and other changes were also found to be associated with specific type of leukemia (Table 1.3). For example, t(15;17) is associated with AML, M3. The breakpoints on the chromosomes are also associated with proto-oncogenes. It is likely that the normal function of the genes are disrupted by the breakages. Chromosome aberrations have diagnostic and prognostic significance.

The third stage involves the use of specific DNA probes to identify genes or chromosome regions. With the introduction of probes labeled with fluorochromes such as painting probes specific for each human chromosome and probes with different fluors for labeling of genes, several abnormalities can be detected simultaneously. This is known as the fluorescence in situ hybridization (FISH) technique. Comparative genomic hybridization (CGH) can be used to detect gain or loss of DNA in a chromosome. All chromosome abnormalities can be identified by spectral karyotyping (SKY) or multicolour FISH (M-FISH). However this analysis identifies only the chromosome involved but not a specific region of the chromosome. Techniques such as Rx-FISH and the use of gibbon DNA labeled with fluors may help to improve karyotyping precision (Rowley, 1999).

1.5.2 Cytogenetic studies at the Institute for Medical Research, Malaysia

The Genetics Laboratory, Division of Hematology, Institute for Medical Research (IMR), Kuala Lumpur, Malaysia was established in 1978. The Genetics Laboratory was the first Laboratory in the country to offer cytogenetic studies as a diagnostic service. The Genetics Laboratory perform peripheral blood lymphocyte cultures to detect chromosome abnormalities in patients with congenital abnormalities, infertility, multiple miscarriages, mental retardation, sex undetermined, etc. The Laboratory also perform bone marrow

cultures to detect chromosome aberrations in patients with hematological malignancies such as leukemia, lymphoma, etc (Chin 1994).

The Genetics Laboratory also provide training in blood and bone marrow cultures for laboratories in Government Hospitals as well as private laboratories throughout the country. The people who come to the Genetics Laboratory for training include pathologists, trainee pathologists, students doing their PhD, Masters and B. Sc., scientists, and medical laboratory technologists. In 1988 the Genetics Laboratory organised a one month training course on peripheral blood culture (from blood culture until banding & staining of metaphase spreads) for all the main peripheral laboratories in the Government Hospitals of each state in the country. After the course, the peripheral laboratories were able to perform cytogenetic studies until the 'banding and staining' stage. The slides were sent to the IMR for interpretation by the Geneticists. All cytogenetic studies involving blood culture is by appointment.

At the moment the Genetics Laboratory, IMR is the only laboratory in the country doing cytogenetic studies on bone marrow for hematological malignancies. The bone marrow specimens are sent to the Genetics Laboratory daily mainly from Hospital Kuala Lumpur (HKL) and Hospital Universiti (UH), Kuala Lumpur. The other government hospitals that sent bone marrow specimen for cytogenetic studies are Hospital Universiti Kebangsaan Malaysia (HUKM), Kuala Lumpur; Hospital Universiti Sains Malaysia (HUSM), Kelantan; Hospital Ipoh, Perak; and Hospital Penang. In 1998 private hospitals such as Lam Wah Ee Hospital and Gleneagles Medical Centre from Penang started to sent bone marrow specimens by courier service to the Genetics Laboratory. Private hospitals within the Klang Valley such as Assunta Hospital, Pantai Medical Centre, Ampang Puteri Specialist Hospital also send bone marrow specimens to the Genetics Laboratory.

Cytogenetic studies can be used for the diagnosis of the type of hematological malignancy. It can be used for the monitoring of the progression of the disease. For example, additional chromosomal abnormalities in CML patients during the course of the disease in patients who only had a Ph chromosome at diagnosis indicates impending blast crisis. Cytogenetic studies can also be used to monitor the success of chemotherapy and

BMT. When patients go to remission after chemotherapy a normal karyotype is seen (except Ph+ CML patients). After a BMT the presence of the donor karyotype indicates engraftment (if donor and recipient are of opposite sex). It. can also be used for the prediction of the prognosis of the disease. For example, t(8;21) in AML, M2 indicates a good prognosis.

I have been attached to the Genetics Laboratory doing cytogenetic studies as a diagnostic service since I joined the IMR in April 1980 as a research officer (geneticist). My other main duties include research, training and laboratory management. I will present cytogenetic findings in all newly diagnosed CML patients (untreated patients) whose bone marrow has been sent to the IMR for cytogenetic studies, as the first part of my PhD thesis. The second part of my PhD thesis is on molecular studies in CML.

4.1.1 Basic cytogenetic laboratory procedures

This topic will be discussed in relation especially to bone marrow culture. Traditional cytogenetic studies require cells that are actively dividing. Specimens that contain proliferating cells are bone marrow, lymph nodes, solid tissues and chorionic villi. Peripheral blood lymphocytes require the addition of a mitotic stimulant (e.g. PHA, pokeweed) for the cells to undergo mitosis. Chromosomes can be distinguished individually under the light microscope at metaphase. The choice of specimen for chromosome analysis depends on clinical indications of the patient. In patients with hematological malignancies (e.g. leukemia, lymphoma) the specimen of choice is bone marrow aspirate.

a) Specimen collection and handling

Bone marrow specimen must be collected into sterile tubes containing preservative-free, sodium heparin. The first few milliliters of the bone marrow tap contain the highest proportion of cells and are the best sample for cytogenetics studies. Blood dilutes the bone marrow sample in later taps and reduced the number of actively dividing cells. The success of bone marrow culture depends on the number of dividing cells.

Transport specimen immediately at room temperature to the laboratory. If delay is unavoidable store the specimen at 4 ^{0}C and transport in a vacuum flask packed with ice. Bone marrow specimens should be processed immediately upon receipt to avoid cell death. At the Genetics Laboratory, Institute for Medical Research we insist that bone marrow specimens reaches the laboratory on the same day of collection, or the latest the next day.

b) Culture media

Specimens for chromosome studies are grown in aqueous growth media such as RPMI 1640, MEM. Culture media are balanced salt solutions containing salts, glucose, and buffering system to maintain the proper pH. Culture media that are obtained commercially are usually incomplete. They have to be supplemented with L-glutamine, fetal bovine serum (FBS) and antibiotics. For bone marrow culture, PHA is not added.

L-Glutamine is an amino acid essential for cell growth. It is unstable and breaks down on storage to D-glutamine. L-glutamine must be stored frozen to retain its stability. It is best added to the culture medium just prior to its use.

Serum is essential for good cell growth. About 10-30% FBS is usually added to the culture medium. Penicillin/streptomycin, kanamycin, and gentamicin are bacterial inhibitors commonly used in tissue culture media to retard the growth of microorganisms.

Mitotic stimulant such as PHA is not used for bone marrow culture. However, B-cell mitogens (e.g. Epstein Barr virus, pokeweed) can be used to stimulate B lymphocytes of B-cell leukemia and lymphoma. Blood and bone marrow samples which consist of free-floating cells can be cultured in sterile centrifuge tubes or tissue flasks.

c) Preparation of specimen for culture

Whole blood or bone marrow can be added directly to the culture medium or the white blood cells can be separated from the other blood elements and used to inoculate the culture medium. Separation of the white cells can be accomplished by centrifuging the sample or allowing it to rest undisturbed until the blood settles into three distinct layers. The lowest layer consists of the heavier red blood cells, the top layer consists of

plasma, and the narrow middle layer, the buffy coat consists of the desired white cells. Remove the buffy coat and resuspend in culture media.

Bone marrow cells are cultured at 37 ^{0}C in an incubator. They can be harvested directly, without any time in culture, or a 24-h to 48-h culture time to increase mitotic index. Longer culture periods are generally not advisable because the abnormal cancer cells may be lost over time or be diluted out by normal precursor cells that may be present. In our Genetics Laboratory we usually perform an overnignt culture for bone marrow specimens.

d) Cell harvest

After the cell cultures have been grown for the appropriate period of time and there is sufficient number of dividing cells, the cells are harvested. Harvesting involves collection of dividing cells at metaphase, hypotonic treatment, fixation, the placement of chromosomes on glass slides, so that they may be stained and microscopically examined.

A mitotic inhibitor, colcemid which is an anologue of colchicine is usually used to obtain an adequate number of cells at metaphase. Colcemid binds to the protein tubulin, obstructing formation of the spindle fibers or destroying those already present. This prevents the separation of the sister chromatids in anaphase, thus collecting the cells at metaphase. Exposure time to colcemid is important. A longer exposure results in more metaphases being collected, but they will be shorter because chromsomes condense as they progress through metaphase. Longer chromosomes are generally preferred for cytogenetic studies.

Hypotonic solution is added to the cells after exposure to colcemid. Water enters the cell by osmosis, thus causing the cells to swell. This is critical for adequate spreading of the chromosomes on the slide. Timing is critical. Too long an exposure will cause the cells to burst and too short an exposure will cause clumping of chromsome spreads. Examples of hypotonic solutions are 0.075M potassium chloride (KCl), 0.8% sodium citrate, diluted balanced salt solutions, dilute serum, and mixtures of KCl and sodium citrate. Morphology of the chromosomes is affected by the type of hypotonic solution used.

Fixative (modified Carnoy's solution) containing three parts of absolute methanol to one part glacial acetic acid is used to stop the action of the hypotonic solution and to fix the cells in the swollen stage. The fixative also lyses any red blood cells present in the sample. The fixative must be prepared fresh before use because it absorbs water from the atmosphere.

The fixed cell suspension are dropped onto glass slides. The concentration of the cell suspension can be adjusted to achieve optimal results. A good slide preparation has sufficient number of metaphases that are not crowded on the slide, metaphases that are well spread with minnimal overlapping of the chromosomes, and no visible cytoplasm. Increased temperature and humidity enhance chromosome spreading, whereas cooler temperature and lower humidity decrease it. Longer exposure to hypotonic treatment makes the cell more fragile and increases spreading, but an inadequate exposure can result in cells that are difficult to burst.

Variables in slide preparation include the height from which the cells are dropped; the use of wet or dry slides; the use of cold, room temperature, or warm slides; the use of steam; air –or flame-drying the slides; and the angles at which the slide and/or pipette is held. The slides are 'aged' overnight at 60 ^{0}C or for 1 hour at 90 ^{0}C to enchance chromosome banding. Chromosomes can also be 'aged' by brief exposure to UV light (Keagle et al, 1999).

e) Chromosome banding and staining

Chromosome staining and banding techniques are divided into two broad categories: (i) Those that produce specific alternating bands along the length of each entire chromosome, and (ii) those that stain only a specific region of some or all chromosomes.

Techniques that create bands along the length of the chromosome include G-banding (Giemsa banding), Q-banding (Quinacrine banding), and R-banding (Reverse banding). This property enables the positive identification of the individual chromosome pairs and permits the characterization of structural abnormalities.

Banding resolution is an estimate of the number of light and dark bands in a haploid set. The minimum estimate is about 400 bands. Well-banded, moderately high-resolution metaphases are usually in the 500-550 band range, and prometaphase cells can achieve a resolution of 850 or more bands.

G-banding is the most widely used routine banding method. GTG banding (G bands produced with trypsin and Giemsa) is one of the several G-banded techniques. The 'aged' slides are treated with the enzyme trypsin and stained with Giemsa. Besides Giemsa stain, Wright or Leishman stain can be used. A series of light and dark bands are produced which enables the positive identification of each chromosome. The dark bands are A-T rich, late replicating, heterochromatic regons of the chromosomes, whereas the light bands are G-C rich, early replicating, euchromatic regions. The G-light bands represent the most active regions of the chromosomes compared to the G-dark bands which contain relatively few active genes.

Q-banding is a fluorescent technique. Q-banding is similar to G-banding pattern with some notable exceptions. The large polymorphic pericentromeric regions of chromosomes 1 and 16, and the distal long arm of the Y chromosome fluoresce brightly. The distal long arm of the Y chromosome is the most fluorescent site in the human genome. Q-banding is useful to confirm the presence of Y material. The disadvantage of fluorescent stains is that it is not permanent, require the use of expensive fluorescence microscopes and a darkened room.

R-banding techniques produce a banding pattern that is opposite or reverse of G-banding pattern. There are fluorescent and nonfluorescent methods. Many human chromosomes have euchromatic terminal ends that can be difficult to vitualise with standard G-band techniques because the pale telomeres may fade into the background. R-banding is useful for evaluation of these telomeres.

Techniques that stain selective chromosome regions include C-banding (Constitutive heterochromatin banding), T-banding (Telomere banding), Cd staining (Centromeric dot or kinetochore staining), G-11 banding (Giemsa at pH 11), NOR staining (Silver staining for nucleolar organizer regions), and DAPI/DA staining (4,6-Diamino-Phenole-Indole/Distamycin A). C-banding selectively stain the constitutive

hetrochromatin around the centromeres, the areas of inherited polymorphisms present on chromosomes 1,9, 16, and the distal long arm of the Y chromosome. C-banding is useful for determining the presence of dicentric and pseudodicentric chromosomes, and also for studying marker chromosomes. T-banding results in only the terminal ends or telomeres of the chromosomes being stained.

Cd staining produces a pair of dots at each centromere, one on each chromatid. Only the active or funtional centromeres will be stained. Cd staining can be used to differentiate functional from nonfunctional centromeres, to study Robertsonian translocations, ring chromosomes and marker chromosomes.

G-11 banding specifically stains the heterochromatin regions of chromosomes 1, 9, 16, the distal Yq, and the satellites of the acrocentric chromosomes. It is used to differentiate between human and rodent chromosomes in hybrid cells.

Nor staining selectively stains the NORs located on the satellite stalks of the acrocentric chromosomes. The silver stains only the active ribosomal RNA genes. Nor staining is useful for the identification of marker chromosomes and rearrangements or polymorphisms involving the acrocentric chromosomes.

DAPI/DA staining combines DAPI, a fluorescent dye, with distamycin A, a nonfluorescent antibiotic. Both form stable bonds to similar A-T rich, double stranded regions of DNA. Used together, DAPI/DA fluoresces certain A-T rich areas of constitutive heterochromatin in the C band regions of chromosomes 1,9,16, and distal Yq, and the short arm of chromosome 15. DAPI/DA is used to identify rearrangements of chromosomes 15; to confirm variations in the polymorphic regions of chromosomes 1,9, 16, and distal Yq, and to study marker chromosomes with satellites.

1.5.4 Automation in the cytogenetics laboratory

Cytogenetic techniques are labour intensive. Instruments such as robotic harvesters, environmentally controlled drying chambers, and computerized imaging systems have been developed to assist the chromosome laboratory in sample preparation and chromosome analysis (Gersen et al, 1999). Some cytogenetic laboratories use all of these devices, some use one or two, and some do not use any. The Cytogenetics Laboratory at the IMR started to use the computerized imaging system in January1998. Except for the imaging system, the other techniques for cytogenetic studies at the IMR are performed manually.

a) Robotic harvesters

Robotic harvesters can perform harvesting of mitotic cells for cytogenetic analysis after the addition of colcemid. The robotic machine can perform aspiration, dispensing of hypotonic solution, and as well as fixative. The incubation times for each step are programmed into a computer that controls the robot.

b) Drying chambers

Spreading of chromosomes is achieved by placing one or more drops of this suspension on a number of slides. Chromosome spreading is controlled by the height from which the suspension is dropped, the temperature and the condition of the spread, humidity and air flow of the surrounding. As the cell suspension dropped on the slides dries, the fixative pulls the cell membrane across the slide, allowing the chromosomes of the cells to spread.

Conditioned controlled chambers where air flow, humidity and temperature can be controlled for routine slide preparation are available commercially.

c) Computerized imaging

The traditional method of karyotyping involves photomicroscopy after the location of suitable metaphases using an England finder. A camera is attached on top of the microscope to take photographs of the metaphase spread. The film and photographs is processed in a dark room. The metaphase spreads are cut and paired. Karyotyping by this procedure is time consuming.

The above process can now be performed using a computerized imaging (fully or semi automatic). An image acquisition subsystem can be used to capture the metaphase spreads. The subsystem consists of a microscope camera adapter, a charged couple device (CCD), a frame grabber and an image capture software.

Dedicated cytogenetic imaging software can perform karyotyping (either automatically or semi-automatically), banding analysis and ideogram display are all now commercially available. Optional components such as metaphase finding, FISH analysis (including M-FISH), CGH analysis can be included.

At the IMR, the computerized imaging system used is the Cytovision. Metaphase spreads are captured in the digital form by the Cytovision satellite capture station. The image is then transferred to the Cytovision workstation where karyotyping is performed. Karyotyping is performed semi-automatically. The geneticists have to check the karyotypes manually to ensure that the chromosomes are correctly paired. A laser printer, Lexmark then produces a hardcopy of the metaphase spread and karyotype. The images consisting of metaphase spreads and karyotypes are stored as digital files on the optical disks.

4.1.1 Quality control and quality assurance

A proper quality assurance and quality control (QA/QC) programme requires that

policies for validation of protocols and reagents, training and credentials of individuals performing chromosome analysis, sample identification, safety for laboratory staff and other compliance issues must all be in place. In the United States of America, laboratories are periodically inspected by various state and federal entities, and most of them have internal regulations and guidelines as well. In Malaysia, there is no QA/QC programme at the moment.

Many steps occur after the specimen arrives at the Cytogenetic Laboratory for chromosome analysis until the generation of the final clinical report. Every laboratory must have a Standard operating procedure (SOP) manual in a QA/QC programme. This document contains requirements for the laboratory to perform chromosome analysis: physical space and mechanical requirements, sample collection, transport, personal experience, credential requirements, personal requirements, and safety and protection requirements for those personnel. It also contains a detailed protocol for every procedure the laboratory performs, training of staff, compliance with the various regulatory agencies that monitor and inspect laboratories, and a section pertaining to QA/QP (McAleer and Gersen, 1999).

The Ministry of Health Malaysia is trying to have all its laboratories accredited and comply with ISO (The International Organization for Standardization) /IEC (The International Electronical Commission) 17025 by the end of the year 2001. This International standard specifies the general requirements for the competence of the laboratory to carry out tests and/or calibrations, including sampling using standard methods, non-standard methods, and laboratory-developed methods. The laboratories in the IMR is also involved in complying with ISO/IEC 17025 International standard.

a) Cytogenetic analysis requirements for neoplastic studies

This includes bone marrow aspirate, unstimulated peripheral blood tumor biopsy or aspirate. Karyotyping is done according to the ISCN (An international system for human cytogenetic nomenclature), 1995. Full karyotyping of 20 cells when possible. All metaphase spreads should be analyzed, and at least two karyotypes should be prepared. If more than one cell line is present, at least one karyotype must be prepared from each cell. The resolution should be at least 400 bands. When there is less than 20 analysable cells and an abnormality has been detected, the number of abnormal and normal cells (if any) is reported. When fewer than 20 cells can be examined and an abnormality is not detected, the number of cells studied is reported and additional procedures (FISH, molecular studies) may be recommended if clinically appropriate. For studies of minimal residual disease or engraftment, additional metaphases may be examined (if availabe).

b) Culture failure

Bone marrow culture failures could be due to:

i) An inappropriate specimen is submitted to the laboratory. This may involve peripheral blood with no circulating blasts being collected instead of bone marrow. It may be due to the wrong specimen tube being collected.

ii) An insufficient specimen is submitted to the Laboratory.

iii) The sample did not contain any living cells. This could be due to delay in sample transport or the specimen was not kept at 4 ^{0}C and transported in ice if delay is unavoidable.

iv) The laboratory suffers from a catastrophic equipment failure.

v) Reagent failure.

vi) Human error (usually unlikely).

In the Cytogenetics Laboratory, IMR factors (i) – (iii) are the main causes of bone marrow culture failure.

1.6 Molecular studies

1.6.1 Introduction to molecular studies

Molecular studies for gene rearrangement can be used to provide evidence of clonality in B-cell or T-cell lymphoproliferative disease and also identify certain chromosomal translocation that are associated with the malignancy.

Some of the techniques used to detect gene rearrangement are Southern blot hybridization, pulse gel electrophoresis and polymerase chain reaction (PCR). I will discuss on the PCR here since this is the technique which I would be using for detection of *bcr-abl* gene rearrangement in CML, that is the presence of the b2a2 and b3a2 transcripts. The presence of either of these transcripts indicate that the patient has CML.

4.1.1 Polymerase Chain Reaction (PCR)

a) Introduction to the PCR

The polymerase chain reaction (PCR) was invented in 1985 by Kary Mullis who was then working for Cetus Corporation in California (Saiki et al 1985). The PCR can amplify a small segment of DNA over a billionfold. The method uses a pair of oligonucleotide primers, each about 15 to 50 oligonucleotides in length designed to hybridize to opposite strands of DNA at sites of several kb apart. In the presence of deoxyribonucleotide triphosphates (dNTPs), DNA polymerase, and buffer the oligonucleotides prime the synthesis of DNA on the DNA template. The PCR is based on the repetition of a number of cycles, each cycle comprising of a set of 3 steps, all conducted in succession under different and controlled temperature conditions (Fig. 1.7):

i) Heat denaturation

The double stranded DNA (template) is denatured by heating to a high temperature which causes the two strands to dissociate.

ii) Annealing of primers

Since the oligonucleotide primers are present in excess over the DNA templates, the formation of the primer-template complex will be favored over the reassociation of the two DNA strands when the temperature is lowered.

iii) Primer extension (Amplification step)

The third step is the synthesis of a strand of complementary DNA (c-DNA), through the 5'to 3' extension of each annealed primer. The 3 steps can be accomplished automatically with a thermal cycler.

Usually 25 to 30 or more cycles are performed. In each cycle, the DNA fragments synthesized in the previous cycles becomes templates for the new DNA synthesis. The DNA fragments accumulate exponentially at a rate of 2^n, where n equals the number of cycles performed. The PCR products are DNA of uniform length that span the distance between the sites of oligonucleotide priming. The ends of each amplified DNA fragment (also known as target sequence product or amplicon) have nucleotide sequences that correspond to that of the oligonucleotide primers

However, the exponential amplification is not an unlimited process. A number of factors act against the process being 100% efficient at each cycle. Their effect is more pronounced in the later cycles of PCR. The amount of enzyme becomes limiting after 25-30 cycles of PCR due to molar target excess. The enzyme activity is also reduced due to thermal denaturation. As the concentration of the target strands increases, it competes with primer annealing by reannealing of target strands (Newton & Gordan 1994, Rapley 1998).

The PCR can amplify DNA fragments that are smaller than 5 kb. For large DNA fragments, PCR is inefficient due to the difficulty of synthesizing DNA of sufficient length from one primer to serve as a template for the other primer in the next amplification cycle.

The PCR has found many applications such as the diagnosis of disease states, species identification, detection of viral or bacterial infection, prenatal diagnosis, and forensic sciences.

b) Components of the PCR

The basic components of the PCR are the DNA template, DNA polymerase, dNTPs, primers, magnesium chloride ($MgCl_2$), and buffer. The various reagents are available commercially and some come in kit form.

(i) DNA template

DNA template are usually provided by the researcher or clinician. In general, lengthy DNA template sample preparations are not required for the PCR to work efficiently. Because of the sensitivity of the technique crude DNA samples may be used as templates. For example, in the amplification of DNA from blood samples a simple boiling step is often sufficient to release the DNA. DNA samples of poor condition can also be analysed as only short intact sequences are required. Paraffin embedded material, or event ancient samples can be analysed.

(ii) Primers

Primers used in the PCR are generally designed using information based on existing sequences of close similarities or evolutionary conserved sequences found by searching genetic databases, such as Genbank/EMBL. Amino acid sequence information may also be used to provide deduced nucleotide sequences from which primers may be designed. In general, primers should have a matched GC content of approximately 50% and must not have the potential to form primer-dimer structures or be self-complementary, both of which adversely affect the PCR (Rychlik 1994). A number of computer programmes are currently available to aid in the process of primer design and optimization of annealing temperatures.

(iii) Thermostable DNA polymerase

During the initial development of the PCR the enzyme used to carry out the extension step was the Klenow fragment of DNA polymerase 1. Since the enzyme is heat labile, fresh enzyme was added during each cycle because the enzyme is denatured by the high denaturation temperature. This made the technique labour-intensive and costly. The introduction of thermostable DNA polymerases has enabled full automation of the PCR technique since only one aliquot of the enzyme have only to be added.

The first and most commonly used thermostable DNA polymerase is the *Taq* DNA polymerase which is isolated from a bacterium *Thermus aquaticus* (*Taq*) found in the hot springs of Yellowstone National Park. *Taq* and its recombinant Amplitaq have high processivity, a 5'-3' exonuclease activity, and an optimum temperature at 72 ^{0}C (Eckert et al, 1990). These two polymerases lacks 3'–5' proofreading exonuclease activity and appears to contribute to misincorporation of nucleotides. A derivative of this enzyme, the Stoffel fragment DNA polymerase, has a higher thermostability and is less sensitive to changes in Mg^{2+} concentration and has no 5' – 3' exonuclease activity. Stoffel fragment polymerase is useful in amplifying G-C rich regions where high or prolonged temperatures are required.

A number of DNA polymerases have been discovered and marketed commercially. Vent^{TM} DNA polymerases isolated from *Thermococcus litoralis* in deep ocean floors is highly thermostable and capable of extending templates in excess of 12 kb pairs, have proof reading ability and higher fidelity compared to *Taq* DNA polymerases. An exonuclease deficient derivative, Vent (exo $^{-}$) ^{TM}, and another version with higher thermostability (Deep Vent^{TM}) are also commercially available.

Pfu DNA polymerase isolated from the hyperthermophilic marine archaebacterium *Pyrococcus furiosus* (*pfu*) has proof reading activity and incorporates radiolabeled nucleotide and analogues efficiently. It is useful for producing radiolabeled gene probes and for performing cycle sequencing techniques. However, the 3' – 5' exonuclease activity of this polymerase can cause modification and degradation of template and primers. Hence, this enzyme must be added last when preparing the reaction. A genetically engineered mutant clone of *Pfu* DNA Polymerase (exo-) with no 3' – 5' exonuclease proof reading activity is also commercially available.

UITma^{TM} DNA polymerase is encoded by a recombinant modified form of the *Thermotoga maritima* (*Tma*) DNA polymerase gene. *Tma* is a hyperthermophilic, Gram-negative eubacterium that was first isolated from geothermally heated marine sediments in Italy.

Tth DNA polymerase isolated from *Thermus thermophilus* (*Tth*), can perform reverse transcription reactions at 70 ^{0}C in the presence of Mn^{2+}. After DNA synthesis and chelation of Mn^{2+}, the polymerase can carry out polymerization of the template. This dual activity of the *Tth* DNA polymerase enables RT-PCR to be carried out in a single tube.

ii) Other components of the PCR

$MgCl_2$ concentration is a critical component and is required by the DNA polymerase for efficient activity and also incorporation of dNTPs in the extension step of the PCR cycle. It also affects the specificity of the primer DNA template interaction and denaturation of the double-stranded DNA template by increasing the melting temperature. Insufficient Mg^{2+} results in low yields whereas in excess it gives rise to non specific products.

Another component is the buffer/salt composition, usually 50mM KCl and 10mM Tris-HCl which enables the pH to be maintained at 8.3 at room temperature. The pH of the reaction is critical. When the reaction is buffered with Tris-HCl, significant changes in pH appeared to be accompanied with changes in temperature.This condition may affect the amplification of long fragments especially (Cheng et al, 1994).

Usually, the PCR buffer and $MgCl_2$ solution is supplied together with the DNA polymerase as a package when we purchase the DNA polymerase. However the PCR buffers, $MgCl_2$ can also be prepared manually according to one's requirements.

c) Hot start

A technique termed 'hot start' has been used to increase specificity of the PCR. It ensures the separation of one or more of the important reagents of the PCR so that all reaction components are mixed after denaturation of the DNA template. PCR using the hot start technique minimizes nonspecific annealing of primers to nontarget DNA sequences and decreases primer oligomerization. Hot start is also useful when using degenerate primers which may misprime oligomerization.

iii) Confirmation of PCR products

Confirmation of the amplified PCR product is usually resolved by agarose gel electrophoresis. The gel is then stained with ethidium bromide (a fluorescent dye that binds DNA) and viewed under ultraviolet (UV) light (Kipps, 1995).

PCR product can also be confirmed by restriction endonucleases (this depends on the presence of a suitable restriction site within the amplified sequence).

PCR may yield false-positive reactions. False priming can occur either on the genomic DNA or on the primers themselves, resulting in amplified fragments containing irrelevant DNA. The amplified product can be further confirmed by Southern Blot analysis of the PCR products followed by hybridization with the appropriate DNA probes, or a second round of PCR can be performed on the amplified DNA fragments using 'nested' oligonucleotide primers that correspond to such internal sequence (nested PCR).

e) Contamination problems of PCR

PCR is extremely sensitive. Contamination may result in false or unwanted amplification. Extreme care must be taken to avoid contamination. Potential sources of contamination may be due to DNA included during sample preparation, DNA from previously amplified products which may be found in laboratory surfaces, pipettes and even aerosols. It is recommended that PCR reactions be set up in a designated location within a laboratory, but is physically separated from DNA extraction areas. Dedicated equipment, such as pipettes and microcentrifuges, are also desirable. Suitable positive and negative controls should be implemented (Rapley, 1999).

f) Inhibitors and enhancers of PCR

PCR can be inhibited or enhanced by many different substances. Heparin should not be used for the collection of blood for it is a potent inhibitor of PCR. To prevent coagulation, it is best to collect the blood in EDTA. Porphyrin compounds found in blood, are also strong inhibitors of PCR. Porphyrin can be eliminated by lysis of red blood cells and centrifugation to pellet the white cells. Other inhibitors include SDS, proteinase K and phenol which are routinely used in nucleic acid extraction.

Compounds that enhance PCR include formamide, dimethylsulfoxide, polyethylene glycol, glycerol, and DNA-binding proteins. However this has to be determined empirically for each individual amplification reaction.

4.1.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

PCR requires a DNA template and cannot amplify single-stranded RNA.

However, a cDNA (complementary DNA) template can be generated from RNA by using an enzyme called reverse transcriptase. In the presence of dNTPs, and an oligonucleotide primer designed to hybridize to a sequence at the 3' end of an RNA molecule, reverse transcriptase catalyzes the synthesis of a DNA copy (cDNA) from the RNA template. The cDNA can then serve as a DNA template for PCR.

RT-PCR has a number of applications but it is especially useful for analyzing the transcriptional activity of genes and gene isoforms. The technique can quantify the expression of mRNA, analyse differential gene expression and clone cDNAs without constructing a cDNA library. Standard PCR amplifies the complete DNA sequence. PCR is only efficient in amplifying DNA fragments less than 5kb in length. The complete DNA sequence containing introns and exons, being more than 5kb in length may not be suitable for PCR. RT-PCR will produce a shorter product resulting from splicing of the intron sequence in the RNA message and hence in the cDNA. So PCR can be performed on the cDNA instead of the original DNA.

4.1.3 Detection of fusion genes by RT-PCR

RT-PCR can be used to detect fusion genes as a result of chromosome translocation. Fig. 1.8 shows the generation of a fusion gene due to translocation between chromosomes A and B. This positions gene 1 and gene 2 on the same chromosome. The distance separating the two genes is quite large (> 20kb), making it impossible to detect the translocation by PCR of genomic DNA or Southern blot hybridization. The fusion gene is then transcribed into RNA. Splicing and processing of the nuclear RNA into messenger RNA (mRNA) removes the intervening nucleotide bases between gene 1 and 2. The enzyme reverse transcriptase is then used to generate a cDNA template from the mRNA and the PCR is then performed to detect gene rearrangement due to chromosomal translocation (Kipps, 1995)

RT-PCR of total RNA or mRNA can be performed together in one single tube This one step RT-PCR is sensitive, quick and the analysis is reproducible as well (Miller and Storts, 1995). The Acess RT-PCR system supplied by the manufacturer, Promega uses *AMV* reverse transcriptase (*AMV* RT) from *Avian Myeloblastosis* virus for first strand DNA synthesis, and the thermostable *Tfl* DNA polymerase from *Thermus flavus* (Kaledin et al, 1981) for second strand cDNA synthesis and amplification.

The one step RT-PCR procedures reduces the chances of contamination and simplifies the procedure as there is no necessity to aliquot the cDNA synthesized into another tube for PCR. The synthesis of cDNA at elevated temperature (48^{o}C) and *AMV/Tfl* buffer supplied minimizes problems encountered with secondary structures in RNA (Evans & Sillibourne, 1996).

1.6.5 Detection of gene rearrangement studies in CML

Bcr-abl gene rearrangement in Ph+ CML results in the formation of two types of mRNA transcripts, b2a2 and b3a2. For the detection of the transcripts, RNA is isolated from the leukemia cells, reverse transcribed to cDNA followed by PCR. The amplified PCR products can be verified by Southern Blotting followed by probing, or a second

round of PCR (nested PCR) or by DNA sequencing. Among the laboratories involved in *bcr-abl* gene rearangement studies, the procedures for RNA extraction, PCR conditions (oligonucleotide primers, temperature, polymerase enzymes, etc) differs (Martiat et al, 1990; Costello et al, 1995; Lee at al, 1996; Reiter et al, 1998).

I will describe the principles involved in the protocol which I have developed. Full details of the methodology will be described in Chapter 2. Extraction of RNA from white cells is by using trizol LS reagent according to the procedure given by the manufacturer, GibcoBrl. Table 2.2 shows the sequences of the oligonucleotide primers used (Cross et al, 1993). A one step RT-PCR is performed using the Promega Access RT-PCR system. The first pair of primers used for the first round of PCR (35 cycles) are NB1+ and Abl3 which amplifies the region between *bcr* exon b1 and *abl* exon a3 of the fusion *bcr-abl* gene. The amplified PCR products are subjected to a second round of PCR (nested PCR, also 35 cycles). The set of primer pairs used that are internal to the first set ('nested' primers) are: B2A and CA3 for detection of the b2a2 and b3a2 transcripts, and CA3 and A2 which is the *abl* control amplifying the *abl* region not involved in the translocation (Evans & Sillibourne, 1996). Fig. 1.9 shows a schematic diagram of the nested PCR to detect the *bcr-abl* gene rearrangement in CML.

The sensitivity of this test is that after one round of PCR it is possible to detect one Ph+ cell in a background of 10^3 normal cells. A second round of amplification (nested PCR) increases the sensitivity to one Ph+ cell to 10^6 normal cells (Roth et al, 1989).

Bcr-abl gene rearrangement studies can be used for the diagnosis of CML. Cytogenetics study may sometimes not yield any metaphase spreads for analysis. The bone marrrow of some CML patient may be myelofibrotic and hence bone marrow aspirate could not be obtained. Molecular study here has an advantage over cytogenetics study is that peripheral blood can be used if bone marrow is not available (Cytogenetic studies require bone marrow aspirate).

Bcr-abl gene rearrangment studies in CML can be used for differenting Ph+ bcr+ and Ph+ bcr- ALL. The study can also be used for monitoring of the disease such as the detection of Ph chromosome after α-inteferon therapy and detection of minimal residual disease after BMT.

4.2 Objectives of study

My PhD thesis consists of two parts. The first part of my PhD thesis is on cytogenetic findings in CML patients at diagnosis of the disease from January 1997 to December 1999 (five year period). Bone marrow aspirate of these CML patients have been sent to the Genetics Laboratory, IMR for cytogenetic studies.

The second part is on molecular studies of CML. Base on the cytogenetic findings, blood from Ph+ CML patients would be used to develop a protocol to detect *bcr-abl* gene rearrangement studies, that is the b2a2 and b3a2 transcripts.

The objectives of the study are as follows:

1. Cytogenetic studies in CML
   a. To correlate the sex, age and ethnic distribution of CML patient to the disease
   b. To determine the frequency of Ph chromosome in CML at the diagnosis of the disease

c. To determine the frequency of standard and variant translocation
d. To determine the frequency of other chromosomal abnormalites in Ph+ CML at diagnosis of the disease

II. Molecular studies in CML
   a. To develop a protocol to detect *bcr-abl* gene rearrangement in Ph+ CML (the b2a2 and b3a2 transcripts)
   b. To determine the frequency of the b2a2 and b3a2 transcripts in Ph+ CML

Table 1.1 Pluripotential or hemopoietic stem cell disorders (Lichtman, 1995a)

1. *APLASIA OR SUPPRESSION OF STEM CELLS*
Polyclonal aplastic pancytopenia (aplastic anemia)

Table 1.2 Major routes of cytogenetic evolution in chronic myeloid leukemia blast crisis (Block, 1999)

Additional change	Frequency (%)
+Ph*	15
i(17q)	12
+8	11
+Ph, +8	8
+8, i(17q)	7
+Ph, +8, +19	5
+Ph, +19	4
+8, +19	2
+Ph, +8, i(17q)	2
+19	1
i(17q), +Ph	1
+8, i(17q), +19	1
+Ph, +8, i(17q), +19	1
i(17q), +19	>1
i(17q), +19, +Ph	>1

*
+der(22)t(9;22)(q34.1; q11.2)

Disease	Chromosomal Abnormality	Frequency		Genes involved
		Disease Overall %	Specific FAB subtype %	
CML	**t(9;22)(q34;q11)**	**95**	**100**	***bcr-abl***
CML blast phase	**t(9;22) with +8, +Ph, +19, or I(17q)**		**~70**	***bcr-abl***
AML, M2	t(8;21)(q22;q22)	12.8	20	*eto-aml*
AML,M3, M3V	t(15;17)(q22;q21)	12	~95-100	*pml-rara*
AML, M4Eo	inv(16)(p13q22) or t(16,16)(pp13;q22)	8.7	~25	*myh11-cbfb*
AML, M4/M5	t(9;11)(p22;q23)	7.5	~38	*af9-mll*
	t(10;11)(p11-p15;q23)			?-*mll*
	t(11;17)(q23;q25)			*mll*-?
	t(11;19)(q23;p13)			*mll-enl*
	Other t(11q23)			*mll*
AML	+8	15.2		
	+21	5		
	-7 or del(7q)	15.7		
	-5 or del(5q)	6.0		
	-Y	7.5		
	-t(6;9)(p23;q34)		1.0	*dek-can*
	t(3;3)(q21;q26)	1.0		
	or inv(3)(q21q26)	1.5		*evll*
	del(20q)	3.4		
	t(12p) or del(12p)			

Therapy-related AML	-7 or del(7q) and/or –5 or del(5q)	75	
	t(11q23)	3	*mll*
	der(1)t(1;7)(q10;p10)	2	

Table 1.3 Recurring chromosome abnormalities, frequency and proto-oncogenes involved in myeloid leukemia (Le Beau & Rowley, 1995)

Legend:

AML: Acute myeloid leukemia
CML: Chronic myeloid leukemia
V: Variant
Eo: Eosinophilic

CHAPTER TWO

MATERIALS & METHODS

2.1 Sample collection

Cytogenetic studies is done on the bone marrow aspirate of patients with hematological malignancies (e.g. leukemia, lymphoma). This study is a specialised diagnostic test. Clinicians are advised to refer to the IMR Handbook for collection and transport of bone marrow specimens. Clinicians are required to fill up the Cytogenetics request form (Appendix 1) for chromosome studies as well. The bone marrow aspirate (about 2ml) is collected in a 10ml sterile tube containing transport media (RPMI 1640, heparin and fetal calf serum) and sent immediately to the Genetic Laboratory, Division of Hematology, IMR. Every specimen sent must be acccompanied by a completed Cytogenetic studies request form. The transport media tubes and Cytogenetic studies request forms can be obtained from the Genetic Laboratory, IMR.

Most of the specimen come from HKL and UHKL. The other hospitals such as HUKM, Kuala Lumpur; HUSM, Kelantan; QEH, Sabah; Hospital Penang, Hospital Ipoh also sent specimen to the IMR for cytogenetic study. Specimen are sent daily (working day only) and immediately to the IMR after collection. Specimen from outside Kuala Lumpur (that is Penang, Sabah and Kelantan) are packed in ice, sent by courier service and reaches the IMR the next day. Cytogenetic studies was performed on a total of 256 newly diagnosed CML patients from January 1995 to December 1999. All the 256 CML patients have not been treated yet.

The unstimulated blasts and bone marrow aspirate are put in culture media for 24 hours for cell division. The mitotically active blasts are treated with colcemid and hypotonic solution, fixed, dropped on to slides and air dried. The metaphase spreads are

banded with trypsin, and stained with Leishman stain. Karyotyping of chromosomes is done using an image analyser.

Molecular studies in leukemia is part of my research project entitled 'Gene rearrangement studies in leukemia'. The project is funded by the Ministry of Science, Technology and Environment, Malaysia under an Intensification of Research Priority Area (IRPA) grant. Approval of grant is based on whether the area of study is a national priority area and also whether the scientific content of the project protocol has been approved by top scientists/ clinicians of the country.

Molecular studies in leukemia is performed on the blood/ bone marrow aspirate. About 2 ml of peripheral blood of Ph+ CML patients (confirmed by cytogenetic studies) from UHKL and HKL is collected into a 2.5 ml EDTA bottle. The specimen is sent to the IMR or I personally go to collect the specimen. A total of 52 blood samples of CML patients was collected for molecular studies from December 1998 until February 2001. Blood from 10 normal persons was used as a control.

4.1 Materials for cytogenetic studies

2.2.1 Reagents

RPMI 1640, Flowlab

Fetal bovine serum (FCS), Flowlab

Penicillin streptomycin (PS), Flowlab

L-glutamine, Flowlab

Hanks balanced salt solution (HBSS), GibcoBRL

Colcemid, GibcoBRL

Bromodeoxyuridine (BRDU), Sigma

Potassium chloride, Sigma

Trypsin, GibcoBRL

Leishman stain, Sigma

Depex, BDH

Acetic acid, BDH

Methanol, BDH

Gurr phosphate buffer tablets, pH 6.8, BDH

Saline tablets, Oxoid

Sodium heparin salt, GibcoBRL

4.1.1 Equipment

Centrifuge, Hermle Z383

Refrigerator, National

Incubator, Forma Scientific

Biohazard Cabinet, Clyde-Apac

Fumehood cupboard, Captair

Hot air oven, Memmert

Microscope, Olympus

Vortex mixer, Thermolyne

Satellite capture station, Cytovision

Image analyser, Cytovision

4.2 Preparation of reagents

4.2.1 Transport Media for collection of bone marrow aspirate

a. Mix the following reagents together: 100ml RPMI 1640, 10ml FCS, 75ul sodium heparin (10,000U/ml)
b. Aliquot 5ml into 10ml plain sterile tubes
c. Label T/M (transport media) and state the date of preparation.
d. Store in a freezer
e. Thaw to room temperature before using.

4.2.2 Bone marrow culture media

a. Mix the following reagents together: 85ml RPMI 1640, 15ml FCS, 2ml PS, 1.3ml L-Glutamine
b. Aliquot 5ml into flat bottom culture tubes.
c. Label and store in freezer
d. Thaw to room temperature before use.

4.2.3 Colcemid 5ug/ml

a. Comes in 20mg lypholised form
b. Add 20mg to 100ml HBSS (stock solution)
c. For 5ug/ml dilute as follows: 1 ml stock solution : 3 ml HBSS
d. Store in freezer.

4.2.4 Bromodeoxyuridine

a. Comes in a vial, 250mg
b. Dissolve in 20ml PBS
c. Add 4ml of (b) to 21ml PBS and sterilise by membrane filtration.
d. Aliquot into cryovials (2ml each) and store frozen. Label tube as 'BRDU'
e. For working solution, add 2ml BRDU of (d) to 1 ml colcemid (10ug/ml) and 2ml RPMI. Discard after 2 weeks. Add 100ul to bone marrow cultures labeled BONC.

4.2.5 Sodium heparin salt

a. Dissolve in RPMI 1640 to 10,000 units/ ml
b. Aliquot 1 ml into cryovials. Store at –20 ^{0}C.

4.2.6 Potassium chloride (KCl), 0.075M

a. Weigh 5.5913g KCl
b. Make up to 1 litre with distilled water
c. Store at room temperature

4.2.7 Leishman stain

a. Put glass beads and a magnetic stirrer into a conical flask.
b. Weigh 5g Leishman powder and put into the conical flask
c. Add 2.5 litre methanol and mix
d. Put the flask on the magnetic hot plate and heat at 50 ^{0}C for 60 minutes
e. Leave it in the dark for at least a week
f. Filter before use.

4.3 Methodology for Cytogenetic studies

Note: For cytogenetic studies all chemicals, culture tubes, containers and glassware for cell culture must be sterile. Preparation of reagents for cell culture and dispensing of cells is done in a biohazard cabinet.

4.1.1 Checking of specimen and recording of patients' particulars

a. When samples arrive at the Genetic Laboratory, IMR, check the patient's name written on the container and the Cytogenetic request form. Ensure that the two names are the same.

b. Enter the patient's particulars in to the Bone marrow registration book and index card.

c. Retrieve from the previous case number if the patient has been referred for chromosome studies before.

4.1.2 Cell density calculation (done in biohazard cabinet)

a. When samples reach the Genetics Laboratory, centrifuge the sample at 900rpm for 10 minutes

b. Discard the supernatant

c. Mix the cell pellet

d. Aliquot 1.9ml 2% acetic acid into a tube containing 0.1ml of cell pellet.

e. Place one drop of suspension to one side of hemacytometer.

f. Count all cells in the 16 squares of the hemacytometer using a microscope.

g. Refer to Table 2.1 for amount of cell density to be added to 5ml of culture media.

h. Record the cell density count and amount of sample required per culture.

Note: If the cell density is very high, dilute the cell pellet with plain RPMI 1640 media and repeat the counting. Use the diluted sample for culture.

2.4.3 Setting up of culture

a. The following cultures are set up in order of priority:

i. Overnight culture with colcemid (ONC)

ii ONC with bromodeoxyuridine (BONC)

iii 48 hours with ONC

b. Thaw the bone marrow culture tubes containing 5ml culture media

c. Add the correct amount of cell suspension to each tube

d. Label the tube with the case number and type of culture i.e. ONC, BONC or 48 hours with ONC

e. Incubate the tubes in a 37^{o}C incubator

f. Add 50ul of 5ug/ml colcemid to the tube labeled ONC, 100ul of BRDU to the tube labeled BONC at 4.00 pm

g. Mix well and reincubate at 37^{o}C

Note: Add 50ul of 5ug/ml colcemid to the tube labelled 48 hours with ONC at 4.00pm on the next day of culture.

2.4.4 Harvesting (ONC and BONC)

a. Centrifuge the tubes labeled ONC and BONC for 10 minutes at 900 rpm

b. Discard the supernatent with a pasteur pipette and then resuspend pellet thoroughly

c. Add 8ml of prewarmed 0.075M KCl in an incubator at 37^{o}C, mix well and leave at room temperature for 15 minutes

d. Centrifuge the tubes for 10 minutes at 900 rpm, remove supernatent and mix well.

e. Vortex the suspension to keep the cell pellet moving, then add freshly prepared cold fixative (3 volume ethanol : 1 volume acetic acid) drop by drop to avoid cell clumping. Top up with fixative to 10 ml (done in a fume hood)

f. Centrifuge the tubes for 10 minutes at 900 rpm, pour off the supernatent and resuspend in fixative. Repeat two more times (total of 3 fixations)

Note: Harvesting is done on the second day of culture for tubes labeled 48 hour ONC.

4.1.1 Slide making

a. Gently dilute and resuspend pellet with additional fixative to give a slightly cloudy suspension

b Assess cell suspension using a microscope. If the preparation is too dense, remake slide using a diluted cell suspension. If suspension is too dilute, spin down again and resuspend in less fixative. If the spreads are clumped or in cell membrane, wash with fixative one more time and redrop the slide.

c Apply 2-3 drops of cell suspension onto a clean wet slide and then air dry

d Make 8-10 slides per case

e Store excess cell suspension in fixatives at –20^{o}C. To remake slides, spin down suspension and change fixative once before making slides.

f. Age slides in the oven (56^{o}C) for at least 3 days.

4.1.2 Banding and staining

a. Prepare fresh trypsin solution 0.125% with pH7.3 phosphate buffered saline (PBS).

b. For staining prepare Leishman's stain diluted with 1: 7 phosphate buffer pH 6.8.

c. Prepare four coplin jars each containing the following solutions:

PBS l

Trypsin solution

PBS ll

PBS lll

d. Dip slide in PBS l for l minute

e. Dip slide in trypsin for 5-10 seconds (variable)

f. Rinse slide in the 2 coplin jars containing PBS ll and PBS lll.

g. Stain immediately with fresh Leishman's stain for 4 minutes

h. Rinse in running water and air dry.

i. Mount a coverslip on the slide and check under microscope for chromosome bands. If under banded increase the trypsin treatment timing. If it is overbanded decrease the trypsin treatment or the PBS timing, whichever is appropriate.

j. Screen for good quality metaphase spreads.

2.4.7 Screening of metaphase spreads

a. Each banded slide is screened for well banded metaphase spreads using a bright field microscope. Record the position of the metaphase spreads using a England finder.
b. Screen and record the position for at least 20 good metaphase spreads

2.4.8 Capturing of metaphase spreads

a. Capture at least 10 metaphase spreads using a satellite capture station.
b. Transfer the image to an image analyser.

4.1.1 Karyotyping of chromosome spreads

a. Key in the patients' particulars in the image analyser
b. Karyotyping is performed using an image analyser.
c. Human cytogenetic nomenclature used in reporting is according to ISCN (1995).

4.1.2 The Cytovision system

The Genetic Laboratory, IMR has three Cytovision satellite capture stations and

two Cytovision karyotyping workstation. One of the karyotyping workstation can also capture images for both brightfield and fluorescent.

A . Cytovision karyotyping workstation

The Cytovision karyotyping workstation is designed to meet the requirements of laboratories using brightfield and fluorescent techniques. The system acquires, processes and prints images through its easy-to-use command interface. Communicaton with the system is mediated by means of the mouse, the keyboard and monitor. Information is displayed in standardized formats.

a. Preparing the Cytovision karyotyping workstation for use.
1. Start Cytovision by switching on the power to all the components of the Cytovision.

Turn on power switch leading to power strip for Cyto 1 workstation

Turn on power switch for Cyto 2 workstation

Turn on power switch for Lexmark laser printer.

2. Once the power comes on, the Cytovision workstation will follow its pre-set boot-up and check procedures.

   Once finished, a prompt will appear on the screen

   Console Login:

   Use the keyboard to enter C*yto* and press the *Enter* key.

3. Once the correct user name has been entered the display screen will go blank, except for a flashing cursor located at the top left hand corner of the monitor. The start-up sequence for the Cytovision system will then continue. After a moment, the Cytovision programme will appear displaying Casebase screen.

4. Proceed with the usage of the Cytovision. If in doubt, refer to the '*The Cytovision System User Manual, Software Version 3.5, Dec 1, 1997'* for further details.

a. Exit and Shutting Down Cytovison

   The exit push-button can be used to leave the Cytovision programme and return to a text mode. From here a user may then log out of the system and instruct the Cytovision to prepare for shutdown. It is best to shut down any time you are experiencing a power problem or are concerned about leaving the system unattended.

   As a reminder, networked Cytovision workstations should be brought up and down together. If you are experiencing problems on one station, bringing it down and back up should not affect other stations, as long as they do not attempt to access data or devices (printers, etc) found on the station.

How to do it:

1. Click on EXIT in the Title window.
   - If an image has been modified, the Analysis window appears and the following prompt displays:

This image has been modified – save changes?

-if more than one image has been changed, the colour bar of the display area containing the relevant image flashes for identification. Click *Yes* or *No* as required. The selected image clears for view. If more than one image was changed, the prompt repeats itself for each image.

-The Cytovision station software and the X window System graphical user interface shutdown.

1. The following prompt appears:

cyto:/user2/cyto>

Type *exit*, then press *Enter*.

2. At the login prompt type: *powerdown* then press Enter.
   - If you are asked for a password press *Enter* again. A pre-set shutdown sequence begins.

1. The system ask:

Do you want to start an express powerdown [y, n, q]

Type *y*.

2. The rest of the powerdown process may take several minutes. When the Cytovision completes its shutdown the following message appears:

   The system is down.

   Press any key to reboot.

3. Switch the Cytovision workstation components off in the manner in which they were turned on.

B. Brightfield capture

a. Prepare the Microscope

1. Turn on power switch for microscope
2. Make certain the microscope is equipped with a green filter for G-banded chromosomes.

3. Put the slide on the microscope stage. Under a 10X power objective to locate and focus a G-banded chromosome spread. Choose a metaphase that is overall well spread, although a couple of touching and overlapping chromosomes are still acceptable.
4. Center and focus the specimen at the microscope.
5. Center and focus the condenser (Koehler Illumination). When complete, open the field diaphragm of the microscope beyond the field of view, but not so far as to generate a hot-spot within the image.
6. Switch over to a 100X oil immersion objective. Place a drop of oil immersion on the slide. Center and focus the selected chromosome spread.
7. If the high power objective lens has a diaphragm, open it completely. If it is closed even slightly, resolution may be lost from the G-banded chromosomes.
8. After use, turn off power switch for microscope.

C. Cytovision satellite capture station

The Satellite capture is designed to allow the user to make more efficient use of the main Cytovision workstation by allowing time consuming metaphase capture to be carried out on the Satellite capture station(s). The images are sent, via the network, to the Cytovision workstation to await import when convenient. This enables the use of sophisticated analysis and presentation tools available on the Cytovision to be maximized. All operations of the Satellite capture station are controlled using the *left* mouse button, with tests entered via the keyboard.

a. Getting started

1. Turn on power switch for Satellite capture station and the monitor.
2. Turn the camera on at the power supply box.
3. The system will boot directly to the MS Window based SatCAP programme. Two message boxes will appear.

The first details default network name and address of the host Cytovision workstation.

The second message box will display the ‘Username’ sat and ask for a password.

Type *satcap1* and click *OK*.

The programme will then take you to the SatCAP screen. Along the top you will see the following toolbar:

Cases	Transfer Setup	Help	Exit

The toolbar remains at the top of the screen throughout SatCAP operation and is the access point for the main function screens of the system.

1. Proceed on with further procedures by referring to the *Applied Imaging Cytovision Satellite Capture User Manual*.

a. Quitting SatCAP

1. If the cases or transfer Setup screens are open, they must be closed before exiting SatCAP.
2. When you have finished using Satellite Capture, click on *OK* then select *Exit* from the toolbar.
3. A box will appear asking if you really want to quit, click on *OK*. This quits the programme and exits into an MS Windows Programme manager screen.
4. Open the file menu by clicking on the word *File* at the top left side of the screen. Select *Exit Windows*, then *OK* in the message box to end the Windows programme. When the C:> appears it is safe to turn off the machine.

4.1 Materials for molecular studies in CML

4.1.1 Reagents for molecular studies

Ficoll-Paque, Amersham

Saline tablets, pH7, Oxoid

DNAse RNAse free distilled water, GibcoBrl

Trizol, LS Reagent, GibcoBrl

BCP Phase separating reagent, GibcoBrl

Ethanol, BDH

Isopropanol, BDH

Access RT-PCR system, Promega

DNTP Mixture (10mM each of dATP, dCTP, dGTP, dTTP), GibcoBrl

Oligonucleotide Primers (customised), GibcoBrl (Base sequences listed in Table 2.2)

Platinum Taq DNA polymerse, GibcoBrl

Agarose, Electrophoresis grade, GibcoBrl

10x TBE buffer, GibcoBrl

100bp ladder, GibcoBrl

Ethidium bromide solution, GibcoBrl

Loading dye, Amresco

Polaroid film, 667

4.1.2 Equipment for molecular studies

Microfuge, Jouan

Shaking water bath, Firstek Scientific

Refrigerated centrifuge, Hettich

Refrigerator, National

Freezer, -20, Kelvinator

Freezer, -40, Sanyo

Freezer, -70, Nuaire

Electrophoresis system (tank and power pack), Easy-cast

Microwave oven, Sharp

Weighing balance, Sartorius

Thermal cycler (PCR machine), Perkin Elmer

Camera system, Hoefer

UV transilluminator, Hoefer

4.2 Preparation of reagents

4.2.1 RT-PCR Reaction, Tube M

The Access RT-PCR system, Promega is used for reverse transcription and PCR amplification in one tube (labeled 'M'). This is a one tube two enzyme system. The following reagents /enzymes come with the system:

AMV reverse transcriptase, 5u/ul

Tfl DNA polymerase, 5u/ul

AMV/Tfl 5X Reaction buffer

Magnesium sulphate ($MgSO_4$), 25mM

DNTP mixture (10mM each of dATP, dCTP, dGTP and dTTP)

a. Prepare the reaction mixture for 10 reactions as follows (the amount to be mixed has been optimised by me):

DNAse RNAse free distilled water	136ul
AMV/Tfl 5X Reaction buffer, minus Mg	50ul
dNTP Mixture	5ul
Abl3+ (10uM)	**7ul**
NBl+ (10uM)	**7ul**
$MgSO_4$	10ul
Total Volume	215ul

b. Dispense 21.5ul of above mixture into 0.2ml sterile thin wall tubes (for RT-PCR reaction) and label the tubes 'M' (refered to as Tube M). Store frozen at -70^oC.

4.2.2 Nested PCR reaction, Tubes A and B

For nested PCR (the second round of PCR), Platinum *Taq* DNA polymerase, GibcoBrl (referred to as Pt *Taq*) is used. The following reagents supplied by the manufacturer together with the Pt *Taq* are also used:

Platinum *Taq* DNA polymerase	5u/ul
10X PCR buffer, Minus Mg	
Magnesium chloride ($MgCl_2$)	50mM

For nested PCR prepare two reaction mixtures (each for 10 reactions). The reaction mixtures would be labeled A and B. Tube A is for the detection of gene rearrangement in CML that is the b2a2 and b3a2 transcripts. Tube B is a positive control and it amplifies the region of the c-*abl* gene that is not affected by the translocation.

2.6.3 Preparation of Tube A

a. Prepare reaction mixture A (total 10 reactions) as follows (amount used has been optimised by me):

DNAse RNAse free distilled water	188ul
10X PCR buffer, minus Mg	25ul
dNTP Mixture, GibcoBRL	5ul
CA3 (10uM)	**5ul**
B2 (10uM)	**5ul**
$MgCl_2$	10ul
Pt *Taq*	**2ul**
Total Volume	240ul

b. Dispense 24ul of above mixture into 0.2ml sterile thin wall tubes (for PCR reaction) and label the tubes 'A' (refered to as Tube A). Store frozen at $-70^{o}C$.

2.6.4 Preparation of Tube B

a Prepare reaction mixture B (total 10 reactions) as follows (amount used has been optimised by me):

DNAse RNAse free distilled water	188ul
10X PCR buffer, minus Mg	25ul
dNTP Mixture, GibcoBRL	5ul
CA3 (10uM)	**5ul**
A2 (10uM)	**5ul**
$MgCl_2$	**10ul**
Pt *Taq*	**2ul**
Total Volume	240ul

b. Dispense 24ul of above mixture into 0.2ml sterile thin wall tubes (for PCR reaction) and label the tubes 'B' (refered to as Tube B). Store frozen at $-70^{o}C$.

4.3 Methodology for molecular studies

Note: All reagents and consumables must be sterile. For molecular studies all tubes, pipette tips, containers are sterilised by autoclaving. Gloves must be worn throughout the experiment. Use a new pair of gloves for dispensing of reagents and RNA extraction. Dispensing of reagents, RNA extraction and PCR amplification are all done in separate rooms.

4.3.1 Extraction of white cells

a. Dilute 1 ml whole blood with 1 ml 0.9% saline.
b. Layer the diluted blood on top of l ml ficoll-paque in a 15 ml sterile conical centrifuge tube.
c. Centrifuge at 2,500 rpm in a refrigerated centrifuge at $4^{o}C$ for 25 minutes
d. Aspirate the white cell layer into a 15 ml sterile centrifuge tube containing 10 ml 0.9% saline
e. Centrifuge at 1,500 rpm in a refrigerated centrifuge at $4^{o}C$ for 10 minutes
f. Discard supernatent and resuspend cells in 10 ml 0.9% saline
g. Centrifuge again at 1,500 rpm in a refrigerated centrifuge at $4^{o}C$ for 10 minutes
h. Discard supernatent and resuspend cells in 2 ml 0.9% saline
i. Aliquot 1 ml each into cryovial and store frozen at $-70^{o}C$

Note: All blood samples collected are processed immediately upon arrival to the Genetics Laboratory. Blood samples are never left at room temperature for more than four hours to prevent degradation of RNA. All blood samples are kept at 4 ^{o}C before processing.

4.3.2 RNA Extraction

a. Thaw one cryovial containing frozen white cells to room temperture
b. Add 250 ul of the white cells in to a 1.5 ml ependorf tube containing 750 ul trizol
c. Leave for 5 minutes
d. Add 200 ul BCP phase separating reagent

e. Shake the mixture vigorously for 40 seconds and leave for 15 minutes
f. Centrifuge in a microfuge at 13,600 rpm for 15 minutes
g. The mixture separates into 2 phases, an aqueous upper phase and a pink coloured lower phase.
h. Aspirate the upper phase into a 1.5 ml ependorf tube containing 500 ul isopropanol.
i. Mix and leave for 10 minutes
j. Centrifuge at 13,600 rpm for 10 minutes
k. Remove supernatent and resuspend RNA pellet in 1000 ul 70% ethanol
l. Centrifuge at 13,600 rpm for 10 minutes
m. Remove supernatent and air dry the RNA. Do not let the RNA dry completely as this would result in the RNA being difficult to dissolve.
n. Dissolve RNA in 50 ul – 200 ul DNAse RNAse free water
o. Heat the RNA in a water bath at 55-60^{o}C for 10-15 minutes
p. Store at –70^{o}C

4.3.3 One step RT-PCR

a. Thaw tube M.
b. Add 0.5 ul *AMV* and 0.5 ul *Tfl* into tube M
c. Add 2.5 ul RNA to tube M.
d. Incubate in a thermal cycler at the following temperatures:
    i. 48^{o}C for 46 minutes followed by 94^{o}C for 2 minutes
    ii. 35 cycles, each cycle consisting of 3 temperatures: 94^{o}C for 1 min, 64^{o}C for 1 min, 72^{o}C for 1 min

    and

    iii a final extension step, 73^{o}C for 10 minutes
e. A control tube M with *AMV* and *Tfl* but without RNA is incubated in the above reaction in the thermal cycler

2.7.4 Nested PCR

a. Thaw one of each, tubes A and B.

b. Take 1 ul each from the PCR product of tube M and add to tubes A and B

c. Incubate tubes A and B in a thermal cycler at the following temperature:

   i. 96^{o}C for 2 min

   ii. 35 cycles, each cycle consisting of 3 temperatures: 96^{o}C for 1 min, 64^{o}C for 1 min, 72^{o}C for 1 min

   and

   iii a final extension step, 73^{o}C for 10 minutes

d. Store the nested PCR products, tubes A and B at –70^{o}C (if electrophoresis is to be done later)

2.7.5 Agarose gel electrophoresis

a. Prepare 2 % agarose gel by dissolving 0.8 g of agarose in 40 ml 1 x TBE buffer in a microwave oven.

b. Add 1ul ethidium bromide to (a).

c. Pour the melted agarose in a tray with a comb to make the wells (Easy-Cast)

d. When the agarose has solidified, remove the comb and put the gel in a refrigerator at 4^{o}C until further use. Do not keep the gel for more than a day.

e. Put 1 ul of the nested PCR product, l ul of loading dye and 6 ul distilled water on a piece of parafilm. Mix and load onto a well in the agarose gel.

f. For the molecular marker, add 1 ul of 100bp ladder, 10 ul of distilled water and 2 ul of loading dye on a piece of parafilm. Mix and load 6 ul each at the opposite ends of the wells.

g. Run the electrophoresis at 130V for an hour (or until the dye is about three-quarter way of the gel length)

4.1.1 Photography

a. View the gel under UV light using a transilluminator.

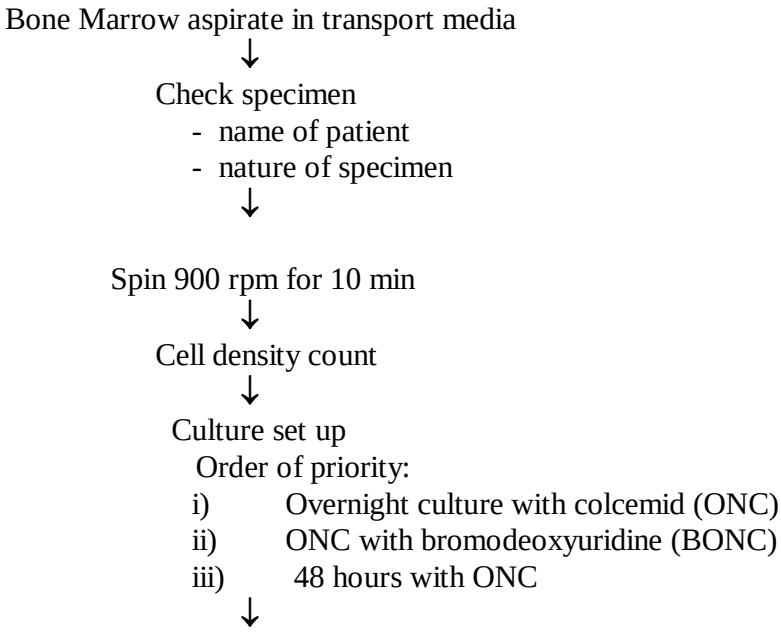
b. Take a photograph of the gel using the camera system.

c. Results are interpreted according to the position of the bands from tubes A and B, with the 100 bp ladder as a molecular marker.

2.8 Flowcharts for cytogenetic and molecular studies

Figs. 2.1 and 2.2 show the flowcharts for cytogenetic studies and molecular studies (*bcr-abl* gene rearrangement studies) respectively, in CML patients.

Fig. 2.1 Flowchart of cytogenetic studies in CML patients



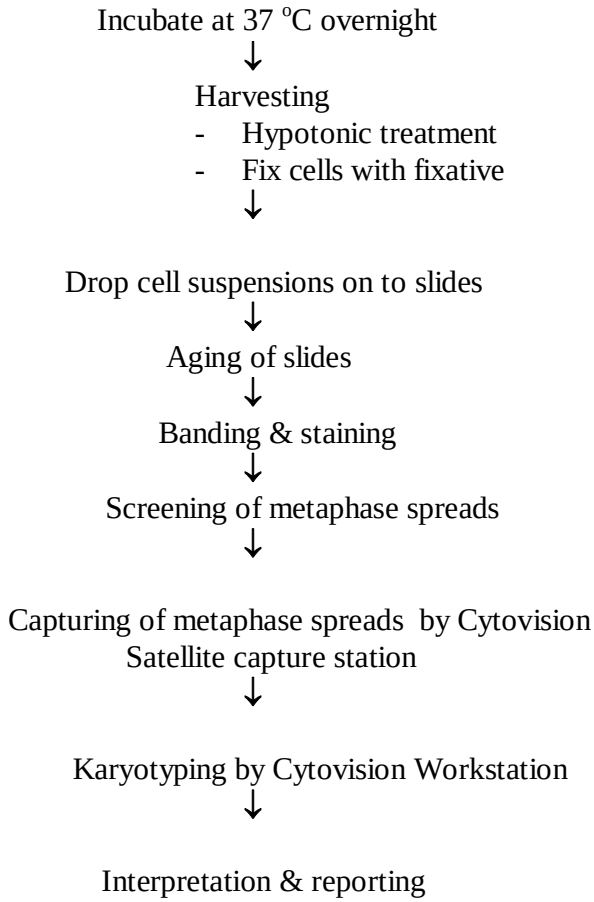
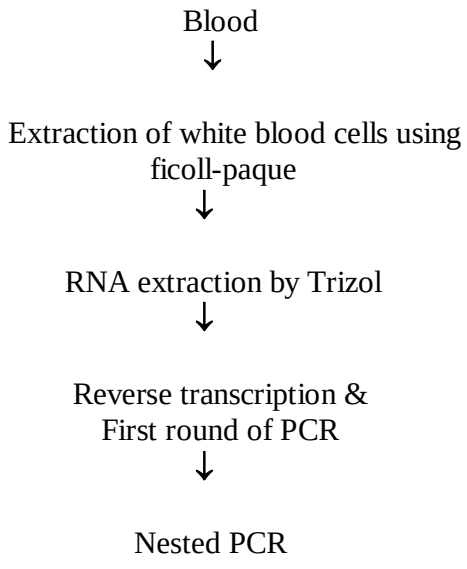


Fig. 2.2 Flowchart of molecular studies (*bcr-abl* gene rearrangement studies) in CML patients



Second round of PCR

↓

Agarose gel electrophoresis
of amplified products

↓

View under UV light & Photography

↓

Interpretation of results

N (when D = 20)	Amount of sample to add (make up to 5mls with medium)	N (when D = 20)	Amount of sample to add (make up to 5mls with medium)	N (when D = 20)	Amount of sample to add (make up to 5mls with medium)
2500	0.01	96	0.26	49	0.51
1250	0.02	92	0.27	48	0.52
833	0.03	89	0.28	47	0.53
625	0.04	86	0.29	46	0.54
500	0.05	83	0.3	45	0.55
416	0.06	80	0.31	44	0.56
357	0.07	78	0.32	43	0.58
312	0.08	75	0.03	42	0.59
277	0.09	73	0.34	41	0.6

250	0.1	71	0.35	40	0.62
227	0.11	69	0.36	39	0.64
208	0.12	67	0.37	38	0.657
192	0.13	65	0.38	37	0.67
178	0.14	64	0.39	36	0.69
166	0.15	62	0.4	35	0.71
156	0.16	60	0.41	34	0.73
147	0.17	59	0.42	33	0.75
138	0.18	58	0.43	32	0.78
131	0.19	56	0.44	31	0.80
125	0.2	55	0.45	30	0.83
119	0.21	54	0.46	29	0.86
113	0.22	53	0.47	28	0.89
108	0.23	52	0.48	27	0.92
104	0.24	51	0.49	26	0.96
100	0.25	50	0.5	25	1.00

Table 2.1 Maglinancy dilution table (Cytogenetic Laboratory, Birmingham Woman's Hospital, England)

N = number of cells in 16 squares of the hemacytometer
D = Dilution factor
Amount of cells to be added is in ml.

Table 2.2 Sequence of oligonucleotide primers (Cross et al, 1993)

Primer	Base sequence
NB1+	5' GAGCGTGCAGAGTGGAGGGAGAACA 3'
Abl3+	5' GGTACCAGGAGTGTTTCTCCAGACTG 3'
B2A	5' TTCAGAAGCTTCTCCCTGACAT 3'
CA3	5' TGTTGACTGGCGTGATGTATTGCTTGG 3'
A2	5' TTCAGCGGCCAGTAGCATCTGACTT 3'

CHAPTER THREE

RESULTS

3.1 Cytogenetic findings in CML patients

From January 1995 to December 1999, cytogenetic studies was performed on a total of 256 newly diagnosed CML patients sent to the Genetic Laboratory, IMR. All the 256 CML patients had not been on treatment yet. The diagnosis of CML was done by the hematologists of the referral hospitals. Out of the 256 CML patients,160 (62.5%) were male and 96 (37.5%) were female. The age of the 256 CML patients ranged from 2 years to 73 years, with a median age of 36 years. 8.2% (21) of the CML patients are children (below 15 years of age). The racial distribution of the CML patients among the various ethnic group of Malaysia are 89 (34.8 %) Malay, 117 (45.7%) Chinese, 37 (14.4%) Indian and 13 (5.1 %) other racial group. The other racial group include mainly the natives of Malaysia. Table 3.1 shows the sex, ethnic group and the referral hospitals of the 256 newly diagnosed CML patients sent to IMR for cytogenetic studies, 1995-1999.

Out of the 256 CML patients, 139 (54.3%) were referred from HKL, 94 (36.7%) UHKL and 23 (9.0%) from private hospitals and other government hospitals outside the Klang Valley. Hence, a majority of the samples (91%) come from HKL and UHKL. In 1998, private hospitals and other government hospitals started to send bone marrow specimen for cytogenetic studies (Table 3.1). The private hospitals include Lam Wah Ee Hospital, Penang; and Gleneagles Medical Centre, Penang. The other government hospitals are HUKM, Kuala Lumpur; Hospital Ipoh, Perak; Hospital Penang; Hospital USM, Kelantan; and QEH, Sabah.

Out of the 256 newly diagnosed CML patients, 222 (86.7%) were Ph+ and 34 (13.3%) have a normal karyotype, that is Ph-. Patients with both Ph+ and Ph- spreads are considered Ph+. Among the 222 Ph+ CML patients, 204 (91.9%) have the standard t(9;22) translocation and 18 (8.1%) have variant translocation (simple and complex). Among the 18 variant Ph, simple variant Ph (involving two chromosomes, that is a chromosome 22 with another chromosome other than 9) was found in nine CML patients and complex variant Ph (involving three or more chromosomes, that is chromosome 9, 22 and other chromosomes) in also nine CML patients. Table 3.2 summarises the cytogenetic findings of all the newly diagnosed CML patients sent to the IMR for cytogenetic studies, 1995-1999. The calculations for percentages of Ph+ vs Ph- CML, standard Ph vs variant Ph, and Ph+ with additional chromosomal abnormalities is shown in Table 3.2A.

Fig. 3.1 shows a normal male karyotype and Fig. 3.1a its metaphase spread. Fig.3.2 shows a karyotype of the standard translocation between chromosomes 9 and 22, t(9; 22) in a CML patient, giving rise to the standard Ph chromosome. Examples of karyotype of variant translocation are shown in:

i) Fig 3.3 : 46, XX, t(7;9;22); Complex variant translocation between chromosomes 7, 9 & 22

ii) Fig. 3.4 : 46, XY, t(17;22); Simple variant translocation between chromosomes 17 & 22

iii) Fig. 3.5 : 45, X, -Y, t(14;22); Monosomy Y, & simple variant translocation between chromosomes 14 & 22

iv) Fig.3.6 : 46, XY, t(18;22); Simple variant translocation between chromosomes 18 & 22

v) Fig.3.7 : 46, XY, t(9;22;21); Complex variant translocation between chromosomes 9, 22 & 21

vi) Fig.3.8 : 46, XX, t(5;9;22); Complex variant translocation between chromosomes 5, 9 & 22

vii) Fig.3.9 : 45, X, -Y, t(10;22); Monosomy Y, & simple variant translocation between chromosomes 10 & 22

and

viii) Fig.3.10 : 46, XY, t(9; 22; 12); Complex variant translocation between chromosomes 9, 22 & 12.

In the newly diagnosed Ph+ CML patients, 33 (14.9%) CML patients (30 with standard Ph and 3 with variant Ph) have additional chromosomal abnormalities besides the Ph chromosome. Additional chromosome aberrations commonly seen are an additional Ph chromosome and trisomy 8. Where monosomy is concerned, monosomy 7 is seen in three of the 34 patients. Two Ph+ male CML patients have monosomy Y. Cytogenetic findings of 30 CML patients with the standard translocation having additional chromosomal abnormalities are shown in Table 3.4. Cytogenetic findings of three CML patients having variant Ph chromosomes with additional chromosome abnormalities are shown in Table 3.3.

Examples of karyotype of variant Ph with additional chromosomal abnormalities are shown in

i) Fig. 3.5 : 45, X, -Y, t(14;22); Monosomy Y, & simple variant translocation between chromosomes 14 & 22

and

ii) Fig.3.9 : 45, X, -Y, t(10;22); Monosomy Y, & simple variant translocation between chromosomes 10 & 22.

Examples of karyotype of CML patients with standard Ph having additional chromosomal abnormalities are shown in:

i) Fig. 3.11 : 46, XX, t(9;22), i(17q); Two cell lines: One with standard Ph chromosome & isochromosome of the long arm of 17, and the other with Ph chromosome only

ii) Fig.3.12 : 51, XY, +6, t(9;22), +10, +13, +19, +der(22), t(9;22) ; Two Ph chromosomes; trisomy 6, 10, 13 & 19

iii) Fig.3.13 : 45, XY, t(7;17)(9;22), -17 ; Monosomy 17 & double translocation: standard Ph, & translocation between chromosomes 7 & 17

iv) Fig.3.14 : 45, XY, t(9;22) t(2;9), -7, der(13), der(16) ; Monosomy 7, derivatives of chromosome 13 & 16, double translocation: Standard Ph chromosome, & translocation between chromosomes 2 & 9.

v) Fig.3.15 : 46, XX, 6q-, -8, t(9;22), + mar; Deletion in the long arm of chromosome 6, monosomy 8, standard Ph & marker chromosomes

vi) Fig.3.16 : 46, XX, del(3p), t(9;22) ; Deletion in the short arm of chromosome 3 & standard Ph chromosome

vii) Fig.3.17 : 47, XX, del(5q), t(9;22), +8 ; Deletion in the long arm of chromosome 5, standard Ph chromosome, & trisomy 8

viii) Fig.3.18 : Ph, add(17p) [10] / 2 Ph, monosomy 13, add(17p) [6]; Two cell lines: One with standard Ph chromosome & additional material on the short arm of chromosome 17; and the other with two Ph chromosomes, monosomy 13 & additional material on the short arm of chromosome 17

and

ix) Fig. 3.19 : 46, XY, t(9;22), t(1;5); Double translocation: Standard Ph chromosome, and translocation between chromosomes 1 and 5

3.2 Molecular findings in CML patients

Molecular studies that is *bcr-abl* gene rearrangement studies was performed on a total of 52 CML patients. All these 52 patients was found to have the Ph chromosome from cytogenetic studies performed by the Genetic Laboratory, IMR. For normal control, gene rearrangement studies was done on ten normal individuals.

After RT-PCR (that is the first round of PCR with primers NB1+ and Abl3) the amplified products were then put into tube A and tube B for a second round of PCR (nested PCR) which consists of 35 cycles also. Tube A contain primers CA3 and B2A (internal to the first set) to detect *bcr-abl* gene rearrangement in CML (Ph chromosome at the molecular level). The presence of a b2a2 transcript or b3a2 transcript indicates *bcr-abl* gene rearrangement in CML. This can be detected by performing an agarose gel electrophoresis on the amplified products together with a molecular marker (100bp ladder). When the gel is viewed under UV light, the b2a2 transcript yields a 385bp PCR product while the b3a2 transcript yield a 456bp PCR product (Fig. 3.20). The absence of the amplification product when using primers CA3 and B2 indicates a patient that is not affected by the *bcr-abl* gene rearrangement.

Tube B contains oligonucleotide primers CA3 and A2, which amplifies the region of *abl* that is not involved in the translocation. The result is a 275bp PCR product (Fig.3.20). This *abl* control amplification is considered a positive control for the PCR reaction. The presence of this 275bp product indicates that your PCR reaction is working.

Out of the 52 Ph+ CML patients studied 19 (36.5%) patients had b2a2 and 33 (63.5%) had b3a2 transcripts. The b2a2 transcript is indicated by a nested PCR product of 285kb in length and this is seen as a band between 200bp and 300bp using the 100bp ladder as a molecular marker in the agarose gel electrophoresis. The b3a2 transcript is indicated by a PCR product of 456bp in length. The band lies in the region between 400bp and 500bp of the 100bp ladder. All the 52 CML patients also showed the presence of a 275bp PCR product using the *abl* control amplification, and this is indicated by a band between 200bp and 300bp using the 100bp ladder as a molecular marker. No amplification product was detected in the ten normal control. All the normal control yield a 275bp PCR product with the *abl* control (Fig. 3.20).

As controls, tubes with all the reaction mixture except RNA was amplified in a thermal cycler together with those with RNA. No amplified product was detected after the second round of PCR with both the *bcr-abl* gene rearrangement and *abl* control tubes.

Of the 52 Ph+ CML patients studied, 51 have the standard t(9;22) translocation and one had a variant translocation, t(18; 22), Fig. 3. 6. This patient was found to have a b2a2 transcript.

Table 3.1 Sex, ethnic origin and referral hospitals of newly diagnosed CML patients sent to the Cytogenetics Laboratory, IMR for cytogenetic studies, 1995-1999

Year	Sex		Ethnic Group				Referral Hospita		
	Male	**Female**	**Malay**	**Chinese**	**Indian**	**Others**	**HKL**	**UHKL**	**Other**
1995	20	14	13	14	4	3	23	11	
1996	23	20	12	20	9	2	29	14	
1997	38	18	24	24	5	3	37	19	
1998	42	20	18	33	10	1	26	29	
1999	37	24	22	26	9	4	24	21	
Total (%)	160 (62.5)	96 (37.5)	89 (34.8)	117 (45.7)	37 (14.4)	13 (5.1)	139 (54.3)	94 (36.7)	23 (9.0)

CHAPTER THREE

RESULTS

Figures

13) Fig.3.12: 51, XY, +6, t(9;22), +10, +13, +19, +der(22), t(9;22) ; Two Ph chromosomes; trisomy 6, 10, 13 & 19

14) Fig.3.13: 45, XY, t(7;17)(9;22), -17 ; Monosomy 17 & double translocation: standard Ph, & translocation between chromosomes 7 & 17

15) Fig.3.14: 45, XY, t(9;22) t(2;9), -7, der(13), der(16) ; Monosomy 7, derivatives of chromosome 13 & 16, double translocation: standard Ph chromosome, & translocation between chromosomes 2 & 9.

16) Fig.3.15: 46, XX, 6q-, -8, t(9;22), + mar; Deletion in the long arm of chromosome 6, monosomy 8, standard Ph & marker chromosomes

17) Fig.3.16: 46, XX, del(3p), t(9;22) ; Deletion in the short arm of chromosome 3 & standard Ph chromosome

18) Fig.3.17: 47, XX, del(5q), t(9;22), +8 ; Deletion in the long arm of chromosome 5, standard Ph chromosome, & trisomy 8

19) Fig.3.18: Ph, add(17p) [10] / 2 Ph, monosomy 13, add(17p) [6]; Two cell lines: One with standard Ph chromosome & additional material on the short arm of chromosome 17; and the other with two Ph chromosomes, monosomy 13 & additional material on the short arm of chromosome 17

20) Fig. 3.19: 46, XY, t(9;22), t(1;5); Double translocation: Standard Ph chromosome, and translocation between chromosomes 1 and 5

21) Fig. 3.20: Agarose (2%) gel electrophoresis of *bcr-abl* and *abl* gene fragments after amplification by nested PCR

Table 3.2 Cytogenetic findings in newly diagnosed CML patients sent to the Genetics Laboratory, IMR, 1995-1999.

Year	Standard Ph	Standard Ph + N	Standard Ph + Ab	Variant Ph	Normal	Total
1995	21	4	4	1	4	34
1996	29	4	1	2	7	43
1997	38	-	7	4	7	56
1998	43	2	11	3	3	62
1999	32	1	7	8	13	61
Total	163	11	30	18	34	256

Table 3.2A Calculations on cytogenetic findings in newly diagnosed CML patients sent to the Genetics Laboratory, IMR, 1995-1999.

Total no. of CML patients = 256

i) % Normal spreads vs %Ph+

No. of Ph+ CML = 222
% Ph+ CML = 222/256 x 100 = 86.7%

No. of Normal Karyotype = 34
% Normal Karyotype = 34/ 256 x 100 = 13.3%

For Ph+ CML (222 patients)

ii. % Standard Ph vs % Variant Ph

No. Standard Ph only = 163
No. of Standard Ph + normal spreads = 11
No. of Standard Ph + additional abnormalities = 30

Total with Standard Ph = 163 + 11 + 32 = 206
% Standard Ph = 204/ 222 x 100 = 91.9%

No. Variant Ph = 18
% Variant Ph = 18/222 x 100 = 8.1%

iii % Ph + additional abnormalities

No. of Variant Ph + additional abnormalities = 3 (refer to Table 3.3)
No. of Standard Ph + additional abnormalities = 30
Total no. of Ph positive CML + additional chromosomal abnormalites = 30 + 3 = 33
% Ph positive CML + additional abnormalities = 33/222 = 14.9%

Table 3.3 Cytogenetic findings of variant Ph chromosome found in 18 newly diagnosed CML patients, 1995-1999.

No	Year	Karyotype & Impression	Type of Variant Ph	Figure
1	1995	46, XX, t(19;22) Translocation between chromosomes 19 & 22	Simple	
2	1996	46, XX, t(5;9;22) Translocation between chromosomes 5, 9& 22	Complex	
3		46, XY, t(6;9;22) Translocation between chromosomes 6, 9 & 22	Complex	
4		46, XX, t(3;9;22) Translocation between chromosomes 3, 9 & 22	Complex	
5	1997	46, XY, t(5;22) Translocation between chromosomes 5 & 22	Simple	
6		46, XY, t(9;11;22) Translocation between chromosomes 9, 11 & 22	Complex	
7		46, XX, t(7;9;22) Translocation between chromosomes 7, 9 & 22	Complex	3.3
8	1998	46, XX, t(17;22) Translocation between chromosomes 17 & 22	Simple	
9		46, XY, t(9;22) [22]/ 46,XY, t(7;9;22) [2] Two cell lines: One with standard Ph chromosome only; & the other with translocation between chromosomes 7, 9 & 22	Complex	
10		*Variant Ph & complex abnormalities	Simple	
11	1999	46, XY, t(16;22) Translocation between chromosomes 16 & 22	Simple	

12		46, XY, t(17;22) Translocation between chromosomes 17 & 22	Simple	3.4
13		*45, X, -Y, t(14;22) Monosomy Y & translocation between chromosomes 14 & 22	Simple	3.5
14		46, XY, t(18;22) Translocation between chromosomes 18 & 22	Simple	3.6
15		46, XY, t(9;22;21) Translocation between chromosomes 9, 21 & 22	Complex	3.7
16		46, XX, t(5;9;22) Translocation between chromosomes 5, 9 & 22	Complex	3.8
17		*45, X, -Y, t(10;22) Monosomy Y & translocation between chromosomes 10 & 22	Simple	3.9
18		46, XY, t(9;22;12) Translocation between chromosomes 9, 12 & 22	Complex	3.10

Total No. with simple variant Ph = 9
Total No. with complex variant Ph = 9

* : Variant Ph with additional chromosome abnormalites = 3

Table 3.4 Cytogenetic findings of Standard Ph with additional chromosomal abnormalities in 30 newly diagnosed CML patients

No	Year	Karyotype & Impression	Figure
1	1995	46, XX, t(9;22), +Ph Two Ph chromosomes	
2		46, XY, t(9;22)/ 47, XY, t(9;22), +8 Two cell lines: One with standard Ph chromosome, & the other with Ph chromosome plus trisomy 8	
3		46, XY, t(9;22), 16q- Standard Ph chromosome plus deletion in long arm of chromosome 16	
4		46, XX, t(9;22), t(1;7) Double translocation, standard Ph chromosome & translocation between chromosomes 1 & 7	
5	1996	47, XX, 7q+, t(9;22), +Ph Two Ph chromosomes, plus additional chromosomal material on long arm of chromosome 7	
6	1997	46, XX, t(9;22) / 47, XX, t(9;22), +Ph Two cell lines: one with standard Ph chromosome only, & the other with two Ph chromosomes	
7		46, XY, 7q+, t(9;22) Standard Ph chromosome plus additional chromosomal material on long arm of chromosome 7	
8		46, XX, t(9;22) / 46, XY, t(9;22), -7, +mar/ 46,XY Three cell lines: One with standard Ph chromosome only; the second with Ph chromosome, monosomy 7, & marker chromosome; & the third one with a normal karyotype	
9		46, XX, t(9;22) [18]/ 46, XX, t(9;22), +3q-, -11 [2] Two cell lines: One with standard Ph chromosome only; and the other with Ph chromosome, trisomy 3 with deletion in the long arm, & monosomy 11	

10		45, XY, t(9;22), -18 [16] / 46, XY [3] Two cell lines: One with standard Ph chromosome & monosy 18, and the other with a normal karyotype	
11		47, XY, t(9;22) , +21 [8] / 46, XX, t(9;22) [2] Two cell lines: One with standard Ph chromosome & trisomy 21, and the other with Ph chromosome only	
12		47, XY, t(9;22), +21 Standard Ph chromosome and trisomy 21	
13	1998	46, XX, t(9;22), i(17q) [18] / 46, XX, t(9;22) [2] Two cell lines: One with standard Ph chromosome & isochromosome of the long arm of 17, and the other with Ph chromosome only	3.11
14		51, XY, +6, t(9;22), +10, +13, +19, +der (22) t(9;22) Two Ph chromosomes; trisomy 6, 10, 13 & 19	3.12
15		45, XY, t(7;17) (9;22), -17 Monosomy 17 & double translocation: standard Ph, & translocation between chromosomes 7 & 17	3.13
16		45, XY, t(9;22), t(2;9), -7, der(13), der(16) Monosomy 7, derivatives of chromosome 13 & 16, double translocation: standard Ph chromosome, & translocation between chromosomes 2 & 9.	3.14
17		46, XX, 6q-, -8, t(9;22), + mar Deletion in the long arm of chromosome 6, monosomy 8, standard Ph & marker chromosomes	3.15
18		45, XX, -7, t(9;22) / 46, XX Two cell lines: One with standard Ph chromosome & monosomy 7, and the other with a normal karyotype	
19		46, XY, t(9;22) / 48, XY, t(9;22), +8, +Ph Two cell lines: One with standard Ph chromosome only; and the other with two Ph chromosomes & trisomy 8	
20		47, XX, 7q+, t(9;22), +Ph Two Ph chromosomes and additional material on the long arm of chromosome 7	
21		46, XX, t(9;22) [14] / 46, XX, t(9;22), +Ph [6]	

		Two cell lines: One with standard Ph chromosome only, and the other with two Ph chromosomes	
22		46, XY, 7q+, t(9;22) Standard Ph chromosome & additional material on the long arm of chromosome 7	
23		46, XY, t(9;22), -21, + mar Standard Ph chromosome, monosomy 21, & marker chromosome	
24	1999	46, XX, del(3p), t(9;22) Deletion in the short arm of chromosome 3 & standard Ph chromosome	3.16
25		47, XX, del(5q), t(9;22), +8 Deletion in the long arm of chromosome 5, standard Ph chromosome, & trisomy 8	3.17
26		Ph, add(17p) [10] / 2 Ph, monosomy 13, add(17p)[6] Two cell lines: One with standard Ph chromosome & additional material on the short arm of chromosome 17; and the other with two Ph chromosomes, monosomy 13 & additional material on the short arm of chromosome 17	3.18
27		48, XX, t(9;22), +8, +9, add(2q), del(6p) Standard Ph chromosome, trisomy 8 & 9, additional material on the long arm of chromosome 2, & deletion on the short arm of chromosome 6	
28		46, XY, t(9;22) [3] / 46, XX, -7, t(9;22), +mar [17] Two cell lines: One with standard Ph chromosome only, and the other with two Ph chromosomes	
29		46, XY, t(9;22), t(1;5) Double translocation: Standard Ph chromosome, and translocation between chromosomes 1 and 5	3.19
30		46, XY, t(9;22) [18] / 46, XX, t(9;22), +8 Two cell lines: One with standard Ph chromosome only, and the other with Ph chromosome & trisomy 8	

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Cytogenetic studies in CML

Cytogenetic analysis is an invaluable tool in the diagnosis, prognosis and management of hematological malignancies.The Genetic Laboratory, IMR is the only laboratory in Malaysia that offers cytogenetic studies in hematological malignancies (leukemias, lymphomas, etc). The Laboratory also perform followup cytogenetic studies especially after chemotherapy and bone marrow transplantation to monitor the progression of the disease. All bone marrow specimens sent to the IMR must be accompanied by a Cytogenetic Study Request Form (Appendix 1). Clinicians/ hematologists are required to fill up this Request Form. In my thesis I will only present cytogenetic findings of newly diagnosed CML patients for a five year period from January 1995 to December 1999.

Bone marrow aspirate of 256 newly diagnosed CML patients collected in transport media was sent to the IMR for cytogenetic studies from January 1995 to December 1999. The diagnosis of the disease was done by the hematologists of the hospitals that sent the specimen. The incidence of Malaysian CML patients with the Ph chromosome at diagnosis was 86.7%. The remaining 13.3% CML patients had a normal karyotype. The frequency of Malaysian male CML patients (62.5%) was higher than that of the female (37.5%). The median age of Malaysian CML patients in this study was 36 years.

The incidence of Ph+ CML reported in the literature is 85% - 95%. There is a male predominance in CML and this trend has been observed generally in most type of leukemia throughout the world (Cartwright, 1992). In the Western population the incidence of CML is highest in the age group between 40 – 50 years.

About 91.9% (204) of the Malaysian Ph+ CML patients have the standard t(9;22) translocation and 8.1% (18) have the variant translocation. 50% of variant translocation are simple (involving chromosome 22 with another chromosome other than 9) while the remaining 50% are complex (involving three or more chromosomes, that is chromosomes 9; 22; with other chromosomes). At presentation of the disease, 14.9% of Malaysian Ph+ CML have additional chromosomal abnormalities such as an additional Ph, trisomy 8, monosomy 7, and in males loss of the Y chromosome.

In the Western population about 5-10% of the Ph chromosome are due to variant translocation (simple or complex). High resolution banding demonstrated simple variant translocation to cryptically involve chromosome 9 at the band q34. At diagnosis about 10-20% of the Ph+ CML patients have additional chromosomal abnormalities such as an additional Ph chromosome, trisomy 8 and loss of the Y chromosome (Block, 1999).

Hence, the incidence of Malaysian CML patients having the Ph chromosome at diagnosis, the frequency of standard and variant translocation, a higher incidence of male CML patients than female, and the frequency and type of additional chromosomal abnormalities in Ph+ CML at presentation of the disease is similar to the Western population.

Malaysia is a multi-racial country with three main ethnic groups: Malays (47.2%), Chinese (25.1%) and Indians (7.1%). The other minority ethnic groups (17.6%) comprise of the natives of Peninsular Malaysia, Sabah, Sarawak and non citizens of Malaysia (Yearbook of Statistics Malaysia, 1997). The distribution of CML in this study is as follows; Malays: 34.8%, Chinese: 45.7%, Indians: 14.4% and others: 5.1%. There appears to be a higher frequency of Chinese and Indians having CML than the Malays. Could it be that CML is more common in the Chinese and Indian population than the Malays?

The Chinese and Indian population are mainly concentrated in urban areas and large towns such as Kuala Lumpur (KL), Penang and Ipoh. About 91% of bone marrow specimens for cytogenetic studies are from HKL and UHKL. The remaining 9% comes from Hospital Penang, Hospital Ipoh, two private hospitals from Penang, HUKM, and QEH in Sabah. HKL is our neighbour and UHKL is about 12km from IMR. HUKM is also within the Kuala Lumpur area. Before sending bone marrow aspirate for cytogenetic

studies, the hospitals would have to collect the transport media from IMR. Sending of specimen would not be a problem for hospitals in KL. Hospital Penang and QEH would have to pay for the courier service to collect the transport media and also to send the specimen to IMR. Most of these hospitals have limited budget except for private hospitals where the patients have to pay for every service rendered. The Malay population are concentrated mainly in the East Coast of Peninsular Malaysia and the hospitals there do not request for cytogenetic studies. This could be due to the fact that these hospitals do not even have a hematologist as the government sector is facing a shortage of specialist doctors. So this probably explain why we seem to have more Chinese and Indian with CML.

Another interesting thing to note is that why only these few hospitals request for cytogenetic studies. The hematologists from the two private hospitals in Penang that request for cytogenetic studies were formerly with UHKL. The hematologists from Hospital Ipoh, Hospital Penang and QEH have been attached to Hematology Unit, HKL before being posted to their present hospitals. All University Hospitals like UHKL, HUKM, and HUSM are teaching hospitals (they come under the Ministry of Education, Malaysia) that provide training for medical students to be doctors and the lecturers (hematologists) are aware of the importance of cytogenetic studies.

4.2 Methodology in cytogenetic studies

Traditional cytogenetic studies done in our laboratory also has its limitations. Cytogenetic studies may fail due to the absence of metaphase spreads for analysis. Sometimes the metaphase spreads may be clumped. Bone marrow of some CML patients may be fibrotic and hence bone marrow aspiration would not be available for cytogenetic studies. Hence, there is a necessity to set up other techniques such as molecular cytogenetics and molecular biology studies

An example of molecular cytogenetics is FISH (fluorescence in situ hybridization) which has increased the resolution and application of traditional cytogenetics. FISH is a technique that allows DNA sequences to be detected on metaphase chromosomes, in interphase nuclei, in a tissue section, or in a blastomere or gamete. This technique uses

DNA probes that hybridize to entire chromosomes or single unique sequence genes. The applications of FISH include microdeletion analysis, identification of marker chromosomes, characterization of structural rearrangements and gene rearrangement associated with neoplasia, ploidy analysis for both prenatal and tumor diagnosis, monitoring of unlike gender bone marrow transplants, preimplantation analysis, and gene amplification studies (Blancato, 1999).

Probes for FISH are now available commercially. There are three major categories of DNA sequences used as probes for clinical studies: satellite sequence probes, whole chromosome probes, and unique sequence probes.

FISH can be used to detect *bcr-abl* gene rearrangement on interphase cells. This enables the analysis of many more cells and thus provides details concerning percentages of cells that are positive or negative for the rearrangement. The probes are labeled with different colored fluorochromes, which results in a third fusion color when the translocation brings them into close proximity (Blancato, 1999).

There are many emerging FISH technologies such as comparative genomic hybridization (CGH), 24-color FISH, microFISH, primed in situ labeling (PRINS), fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms (FICTION), and cross species color banding (RxFISH).

The Ministry of Health Malaysia has given approval to the IMR to purchase an Automated Multicolour Chromosome Analysis System costing about RM900,000.00. This system is able to perform brightfield karyotyping, FISH, CGH, RxFISH and Multi-colour (M) FISH. It will be installed at the Genetics Laboratory, IMR in December 2001.

4.3 Molecular studies in CML

Bcr-abl gene rearrangement studies in Malaysian Ph+ CML patients showed the frequency of b2a2 and b3a2 transcripts to be 36.5% and 63.5% respectively. No patient in my study express both b2a2 and b3a2 transcripts. In a study by Reiter et al, 1998 the incidence of b2a2 and b3a2 transcripts in Ph+ CML was 31.6% and 68.4% respectively. Lee et al, 1996 found that the frequency of b2a 2 and b3a2 transcripts in Ph+ CML was 67.9% and 30.2% respectively. One Ph+ CML patient (1.9%) in their study (Lee et al, 1996) was found to express both b2a2 and b3a2 transcripts. Thus in general, the frequency of b3a2 transcripts was found to be higher than that of b2a2 transcripts. There is no difference in the prognosis of patients with b2a2 or b3a2 transcripts.

The advantage of gene rearrangement studies is that it can detect masked Ph chromosome where cytogenetic studies showed a normal chromosome 9 and 22. When cytogenetic studies failed (no spreads) or when bone marrow aspirate cannot be obtained

from the patient, blood can be used for the study. This technique can be used to diagnose CML when the hematologist is not sure whether the patient has CML or some other hematological malignancies (e.g. myelofibrosis). In fact now I have been receiving requests from hematologists in UHKL, HKL and some private hospitals for *bcr-abl* gene rearrangement studies to help in the diagnosis of 'difficult' cases.

Molecular studies in CML have been presented in three conferences, and they are:

i) Chin YM, Zubaidah Z, Teh A, Goh AS, Purushothaman V. Molecular diagnosis of chronic myeloid leukemia. IX Congress of International Society of Hematology, Asian Pacific Division, 24-28 October 1999, Bangkok, Thailand

ii) Chin YM, Teh A, Zubaidah Z. Bcr-abl gene rearrangement in a patient with myelofibrosis. 11th National Biotechnology Seminar, 22-24 November 1999, Melaka, Malaysia

iii) Chin YM, Zubaidah Z, Goh AS, Purushothaman V. Molecular studies in the monitoring of chronic myeloid leukemia. Biology in the Next Millennium, 30 November – 2 December 1999, Kuala Lumpur, Malaysia.

4.3 Methodology in gene rearrangement studies

For RNA extraction, Trizol L-S reagent was used. The procedure is very simple and takes about 3 hours to complete. With Trizol L-S whole blood/ bone marrow can be used. If one is busy, the blood/bone marow can be stored frozen at $-70^{o}C$ until further usage. Hence, there is no necessity to separate the lymphocytes with ficoll-paque. The reason why I separate the lymphocytes is because by doing so the white cells can be stored at $-70^{o}C$ for a longer period of time. The separation procedure takes about 2 hours.

The RT-PCR was conducted in one tube (one step RT-PCR). The advantage is that after RT one need not dispense the cDNA to another tube for PCR. This would minimise contamination. The procedure takes about 4 hours.

About one ul each of the amplified product is then added to two tubes (one to detect the transcript and the other is a positive control) for nested PCR (second round of PCR). The procedure takes about 3 hours.

The amplified product is then subjected to an agarose gel electrophoresis. The gel is then photographed for documentation. The procedure takes about 3 hours. So the

whole process from extraction of white cells to photography takes a total of about 15 hours.

In my laboratory, when the sample arrives I would separate the white cells and store them frozen at –70^{o}C. Extraction of RNA, RT-PCR, and nested PCR would be done on the same day. The next morning, I would do the electrophoresis and photography.

Bcr-abl gene rearrangement in CML can also be detected from DNA studies. I did this procedure for my M. Sc. Thesis. The procedure is as follows: DNA is extracted from the peripheral blood of CML patients. The DNA was digested with *Bgl* II, followed by agarose gel electrophoresis, Southern blotting, hybridization with a 32^{p}-labeled 4.8-kb *phl*/bcr-3 DNA probe, and autoradiography. The whole procedure is very long and tedious and it takes about a week to complete.

That is why nowadays it is better to isolate RNA and perform RT-PCR. As stated earlier my protocol to detect *bcr-abl* gene rearrangement takes about 15 hours to complete. The advantage of RNA is that you need not require a large amount of blood. With nested PCR , the procedure is very sensitive - it can detect one leukemic cell in 10^{6} cells. Hence, this would be useful to monitor MRD, that is detection of *bcr-abl* gene rearrangement after BMT and chemotherapy.

The sensitivity of PCR has its disadvantage too. Since the PCR is capable of ampifying a single copy of DNA, any degree of contamination may result in false or unwanted amplification. This makes the method extremely prone to giving false positive results unless great care is taken when setting up the reactions to avoid any possibility of contamination. The potential sources of contamination not only include positive sample DNA and positive control DNA, but also previously amplified material. These may be found on laboratory surfaces, pipets, and even in aerosals. To overcome these problems preparation of reagents, RNA extraction, and PCR reaction are all done in separate rooms. Separate pipets and pipet tips are used for RNA extraction, preparation of reagents, and PCR amplification (Rapley 1998).

However, the disadvantage of this PCR technique is that it is qualitative in nature – either there is or there is no *bcr-abl* gene rearrangement. Hence, there is a necessity to

develop other techniques such as quantitative PCR to estimate the number of *bcr-abl* transcripts in the CML patients. This technique would require the purchase of a unit of real time PCR machine. Cross et al 1993 found that patients who progress from cytogenetic remission to cytogenetic relapse and then to hematologic relapse had increasing number of *bcr-abl* transcripts in their blood. Conversely, patients without cytogenetic relapse generally had low or falling numbers of transcripts in their blood. Hence, quantitative PCR can be used to monitor the progression of the disease, especially an impending blast crisis.

The Ministry of Health Malaysia has also given the approval to the IMR to purchase A Real Time On-Line Quantitative PCR System costing about RM350,000.00.

This System is able to quantitate mRNA expression or gene dosage levels. It will be installed at the Genetics Laboratory, IMR in December 2001.

4.5 Conclusion

This PhD thesis consists of 2 parts: cytogenetic studies in CML and molecular studies in CML. Cytogenetic studies was performed on a total of 256 newly diagnosed Malaysian CML patients from January 1995 to December 1999. 86.7% were Ph+ and 13.3% Ph negative. In the Ph+ patients, 91.9% have the standard translocation and 8.1% have variant translocation. At presentation of the disease, 14.9% of Ph+ CML have additional chromosome abnormalities. There was also a preponderance of male CML patients in CML. Cytogenetic findings in Malaysian CML patients are similar to that of the Western population. Cytogenetic studies is an invaluable tool in the diagnosis and prediction of prognosis of CML patients.

Bcr-abl gene rearrangement studies in Ph+ Malaysian CML patients showed the frequency of b2a2 and b3a2 transcripts to be 36.5% and 63.5% respectively. This is consistent with the findings in the West that the frequency of b3a2 transcripts is higher than that of b2a2 transcripts. We have successfully developed a protocol to detect *bcr-abl* gene rearrangement studies. Ficoll-paque was used to isolate white cells from the blood of CML patients. Trizol L-S was used to extract RNA from the blood of CML patients. A one step RT-PCR was done using the Acess RT-PCR system from Promega. Primers used were according to the sequence of Cross et al 1993. Nested PCR was performed using Platinum *Taq* DNA polymerase from Gibco. The amplified products were subjected to agarose gel electrophoresis and detected using UV light.

Bcr-abl gene rearrangement studies can be used to diagnosis CML especially when cytogenetic studies failed. The procedure takes about a day compared to cytogenetic studies which takes about a week. Blood can be used if bone marrow is not available. The protocol developed can be used to distinguish between Ph+ bcr+ and Ph+ bcr- ALL. However, the disadvantage of gene rearrangement study when compared to cytogenetic study is that one is unable to detect further chromosome changes in CML which signifies the coming of blast crisis.

Cytogenetic and molecular studies are very useful to the clinicians/ hematologists in the diagnosis, prediction of prognosis, and monitoring MRD after chemotherapy and BMT. These two studies can help the clinicians in the management of their CML patients.

In future, it would be very useful for the Genetic Laboratory, IMR to develop techniques such as FISH, CGH, RxFISH, M-FISH, and quantitative PCR. These techniques can be used as additional tests for the diagnosis, management, and prediction of prognosis of patients with hematological malignancies (such as leukemia) in Malaysia.

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