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DETECTION OF ANEUPLOIDY FOR CHROMOSOMES 7 AND 8 USING FLUORESCENCE *IN SITU* HYBRIDIZATION IN PATIENTS WITH APLASTIC ANEMIA AND SEQUENCING OF THE MITOTIC CHECKPOINT GENE *hBUB1*

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DOCTOR OF PHILOSOPHY

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OLD DOMINION UNIVERSITY
May 2001

Approved by:

Christopher Osgood
(Director)

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ABSTRACT

DETECTION OF ANEUPLOIDY FOR CHROMOSOMES 7 AND 8 USING FLUORESCENCE *IN SITU* HYBRIDIZATION IN PATIENTS WITH APLASTIC ANEMIA AND SEQUENCING OF THE MITOTIC CHECKPOINT GENE *hBUB1*

Laura Jane Aridgides
Old Dominion University, 2001
Director: Dr. Christopher Osgood

Aplastic anemia (AA) is characterized by complete bone marrow failure. Progression to myelodysplastic syndromes (MDS) and acute nonlymphocytic leukemia (ANLL) occurs frequently. At the time of transformation, cytogenetic abnormalities are common. Detection of cytogenetic abnormalities prior to leukemic transformation may indicate future disease progression. Karyotype analysis is the current method of choice to evaluate chromosome aberrations. However, fluorescence *in situ* hybridization (FISH) is more sensitive in detecting these abnormalities.

hBUB1, a mitotic spindle checkpoint gene, was shown to be mutated in two colorectal cancer cell lines with high levels of aneuploidy (Cahill, et al., 1998). Although theoretically possible, conclusive evidence does not currently exist establishing a link between aneuploidy levels and mutations within a mitotic spindle checkpoint

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gene. *hBUB1* is the most characterized of the mitotic checkpoint genes.

FISH was used to detect cytogenetic abnormalities for chromosomes 7 and 8 in bone marrow samples from patients with AA. In addition, ribonucleic acid (RNA) from all patient samples also underwent *hBUB1*-specific reverse transcription polymerase chain reaction (RT-PCR), followed by sequencing of the RT-PCR product. Statistical analyses were performed on FISH and sequencing results. Additional samples from patients with a variety of bone marrow disorders also underwent *hBUB1*-specific RT-PCR and sequencing without FISH analysis.

Seven patient samples out of 46 (15.2%) showed elevated levels of aneuploidy for chromosomes 7, 8, or both. Four of the seven samples showed abnormalities previously undetected by a karyotype analysis. This indicates that FISH analysis is approximately twice as sensitive as a karyotype analysis, and may assist in earlier diagnosis and proper treatment of patients with AA. Statistical analysis showed an increased level of monosomy 8 in African-American males. The age of the patient and responsiveness to treatment did not correlate with the level of aneuploidy. Results from the *hBUB1*-specific RT-PCR and sequencing were inconclusive due to the high probability of Taq-induced PCR

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artifact, however there was no apparent correlation between the presence of aneuploidy and the sequencing results. Seventy-eight to 85% of all patient samples analyzed did not amplify any *hBUB1*-specific RT-PCR product.

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This dissertation is dedicated to my wonderful husband Mark, my family and my friends. Thank you for your never ending support.

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ABBREVIATIONS

AA = aplastic anemia

AL = acute leukemia

AML = acute myeloid leukemia

Amp = ampicillin

ANLL = acute nonlymphocytic leukemia

APC = anaphase promoting complex

ATG = antithymocyte globulin

BUB = budding uninhibited by benzimidazole

CD = cluster of differentiation

CDC = cell division cycle

cdk = cyclin-dependent kinase

cDNA = complimentary DNA

CFU = colony-forming unit

CIN = chromosomal instability

CKI = cyclin-dependent kinase inhibitor

CMML = chronic myelomonocytic leukemia

CsA = cyclosporin A

DAPI = 4′,6-diamidino-2-phenylindole

DNA = deoxyribonucleic acid

EDTA = disodium ethylenediamine tetraacetate

Epo = erythropoietin

FA = Fanconi's anemia

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FAB = French, American, and British

FISH = fluorescence *in situ* hybridization

gal = galactosidase

G-CSF = granulocyte colony-stimulating factor

GM-CSF = granulocyte-macrophage colony-stimulating factor

GPI = glycosylphorphatidylinositol

GVHD = graft versus host disease

IL = interleukin

INF = interferon

IPTG = isopropyl β-D-thiogalactopyranoside

LB = Lennox broth

MAD = mitotic arrest deficient

MBG = molecular biology grade

M-CSF = macrophage colony-stimulating factor

MDS = myelodysplastic syndromes

MI = mitotic index

MIN = microsatellite instability

MPF = maturation (M phase) promoting factor

MPS = monopolar spindle

MTOC = mictotubule organizing center

NaI = sodium iodide

NK = natural killer cell

PBS = phosphate buffered saline

PCR = polymerase chain reaction

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PNH = paroxysmal nocturnal hemoglobinuria

RA = refractory anemia

RAEB = refractory anemia with excess blasts

RAEB-t = refractory anemia with excess blasts in transformation

RARS = refractory anemia with ringed sideroblasts

Rb = retinoblastoma

RNA = ribonucleic acid

RT-PCR = reverse transcription polymerase chain reaction

SAA = severe aplastic anemia

SCF = stem cell factor

TAE = Tris-acetate-EDTA

TBE = Tris-borate-EDTA

TE = Tris-EDTA

TGF = transforming growth factor

t-MDS = therapy-related myelodysplastic syndromes

TNF = tumor necrosis factor

TPO = thrombopoietin

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CHAPTER I

INTRODUCTION

HEMATOPOIESIS

Overview

Hematopoiesis is defined as "the formation and development of blood cells involving both proliferation and differentiation from stem cells (Online Medical Dictionary, 2001)." The stem cells necessary for successful hematopoiesis begin to form in the yolk sac during early gestation, and migrate to the liver and the spleen before settling within the bone marrow. It is in the bone marrow that the majority of hematopoiesis occurs in healthy individuals (Sullivan, 2001). However, most of the stem cells within the bone marrow are in a quiescent state (Jordan and Van Zant, 1998; Sullivan, 2001). A portion of the cells undergo self-renewal, but only a tiny fraction of the cells are differentiating at any given time (Sullivan, 2001).

The model for this dissertation is the journal Cell.

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Stem Cell Differentiation

All cells differentiate from a totipotent stem cell (Peterson, 1995; Sachs, 1996). The totipotent stem cell has the potential to travel down the myeloid differentiation pathway, the lymphoid differentiation pathway, or to undergo self-renewal (Klumpp, 2001; Sullivan, 2001). The mechanism that determines the cell's fate is unclear, but it is thought to be either stochastic (random) or induced by external stimuli (cell to cell interactions and/or cytokines) (Enver et al., 1998). When the decision is made to differentiate, the stem cells become multipotent myeloid or lymphoid stem cells. The multipotent stem cells give rise to progenitor cells called colony-forming units (CFU) (Klumpp, 2001; Sullivan, 2001). The myeloid pathway is further subdivided into the erythroid, megakaryocytic and phagocytic branches. The erythroid pathway ends in the formation of erythrocytes, and the megakaryocytic pathway ends in the formation of platelets. The phagocytic pathway subdivides even further into granulocytic and monocytic pathways. The granulocytic pathway divides once more into the neutrophilic, eosinophilic and basophilic pathways, which end in the formation of neutrophils, eosinophils and basophils,

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respectively. The monocytic pathway ends in the formation of macrophages (Sullivan, 2001).

The lymphoid pathway is also subdivided into the B cell pathway, the T cell pathway and the non-B, non-T cell pathway. The B cell pathway ends in the formation of B cells, and the T cell pathway ends in the formation of T cells. The non-B, non-T cell pathway ends in the formation of natural killer (NK) cells (Peterson, 1995; Sullivan, 2001) (Figure 1). The cluster of differentiation (CD) antigens presented on each cell's surface can assist in identification of the different cell types (Sears, 1997).

The Role of Cytokines in Hematopoiesis

Cytokines can act as growth factor hormones that orchestrate the production of cells during hematopoiesis. Each cytokine acts upon one or more specific cell types. To elicit a response, the cell must have a receptor specific for the cytokine. There are a wide variety of cytokines including, but not limited to: erythropoietin (Epo), thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), tumor necrosis factor alpha

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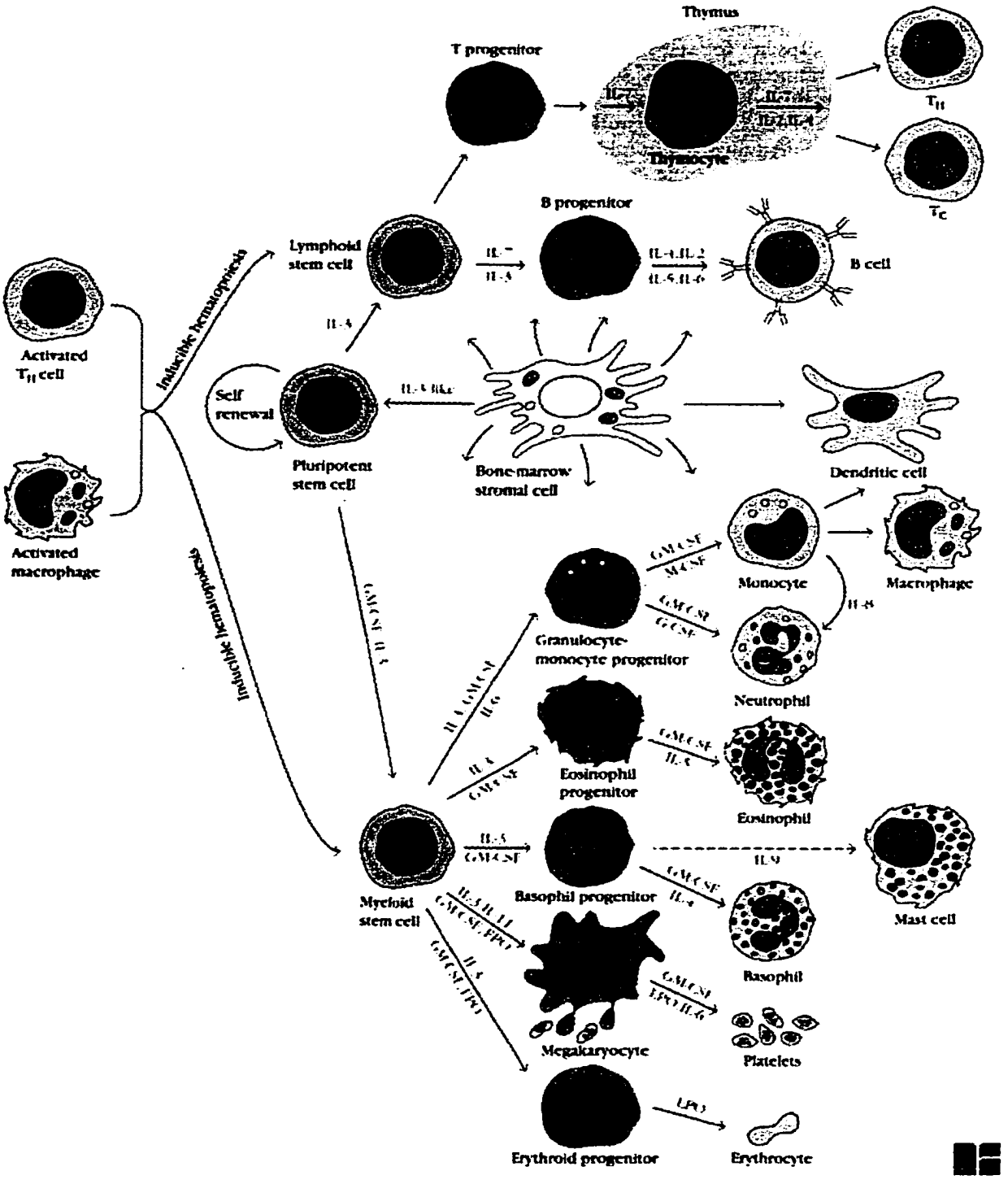

Figure 1. Overview of Hematopoiesis, Including Cytokine Interactions.

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(TNF-α), transforming growth factor beta (TGF-β), and a variety of interleukins (IL) (Klumpp, 2001; Moqattash and Lutton, 1998; Sachs, 1996; Sullivan, 2001). There are cytokines that induce hematopoiesis (such as GM-CSF and IL-2) and there are cytokines that inhibit hematopoiesis (such as TGF-β, IL-10 or interferon gamma (INF-γ)) (Moqattash and Lutton, 1998; Sachs, 1996). The mechanism of stimulation or inhibition may be direct or indirect, and may involve multiple cytokines acting together to achieve the final outcome (Moqattash and Lutton, 1998). A triad comprised of stem cells, cytokines and the bone marrow stroma works together to achieve hematopoiesis (Enver et al., 1998; Moqattash and Lutton, 1998; Orkin, 1996; Sullivan, 2001). The hematopoietic progenitor cells adhere to the bone marrow stroma enabling them to receive cytokine signals from the stroma. Leukemic cells also rely on adhesion to the stroma matrix to increase survival and reduce the likelihood of apoptosis (Greer and Kinney, 1993; Moqattash and Lutton, 1998; Sachs, 1996). A defect in any component of the hematopoietic triad creates the potential for blood-related diseases, such as leukemia (Moqattash and Lutton, 1998; Sachs, 1996).

It is of interest to report on some of the more common cytokines and their actions on normal cell growth; however,

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a complete review is beyond the scope of this manuscript. Epo is involved in erythropoiesis by stimulating differentiation of erythroid colony-forming units (CFU-E) and increasing red blood cell production (Moqattash and Lutton, 1998; Sullivan, 2001). TPO stimulates megakaryocytes and, as a result, increases the number of platelets (Moqattash and Lutton, 1998). GM-CSF in combination with SCF can stimulate various hematopoietic pathways. G-CSF stimulates the differentiation of leukocytes. M-CSF stimulates the differentiation of monocytes. A variety of IL cytokines can assist other cytokines in the stimulation or inhibition of various hematopoietic pathways (Moqattash and Lutton, 1998; Sachs, 1996). There is a tremendous complex cytokine network that has a direct impact on hematopoiesis, of which all functions are not yet known or completely understood.

Cytokines have been implicated in leukemic transformation, which is characterized by uncontrolled cell growth. This can be due to inhibition of apoptosis or an increase in hematopoiesis (Israels and Israels, 1999; Wickremasinghe and Hoffbrand, 1999). Since cytokines are involved in both apoptosis (cell death) and hematopoiesis (inducing cell growth and differentiation), it can be inferred that abnormal cytokine production may contribute

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to the formation of cancer (Israels and Israels, 1999; Moqattash and Lutton, 1998). For example, it has been reported that IL-8 can stimulate *bcl*-2 expression in cells from patients with B-cell chronic lymphocytic leukemia. Increased levels of *bcl*-2 help protect the cancerous cells from apoptosis (an increased level of *bcl*-2 has also been reported in patients with myelodysplastic syndromes (MDS)) (Israels and Israels, 1999; Moqattash and Lutton, 1998; Sachs, 1996; Wickremasinghe and Hoffbrand, 1999). Cytokine effects on leukemic cells are incredibly variable and have been shown to elicit different responses in different patients (Marsh, 2000; Moqattash and Lutton, 1998).

Cytokines have recently been investigated for clinical use in patients with hematological disorders, the caveat being that cytokines can also decrease the effectiveness of some anticancer agents by inhibiting apoptosis (Marsh, 2000; Moqattash and Lutton, 1998; Sachs, 1996; Wickremasinghe and Hoffbrand, 1999). Studies showed that patients with MDS treated with G-CSF and Epo resulted in an increase in hematopoiesis. On the other hand, treatment with growth factors can also inhibit apoptosis of leukemic cells, which is manifested as a poor response rate to some chemotherapeutic agents (Moqattash and Lutton, 1998; Sachs 1996).

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BONE MARROW FAILURE SYNDROMES

Although aplastic anemia (AA) is the primary focus of this manuscript, and is reviewed in detail below, other bone marrow disorders are mentioned in conjunction with AA, and therefore deserve explanation.

Paroxysmal Nocturnal Hemoglobinuria (PNH)

PNH is a clonal stem cell disorder characterized by cells deficient in glycosylphosphatidylinositol (GPI)-anchored proteins (Dunn, et al., 1999; Socié, et al., 2000). The traditional phenotypic marker of dark-colored urine occurs in about half of the patients diagnosed with PNH, and is caused by intravascular hemolysis. Hemolysis occurs due to an increase in compliment (immune-mediated cell lysis) as a direct result of the deficiency in GPI-anchored proteins (Dunn, et al., 1999; Lee, 1993). This deficiency is caused by a somatic mutation in the *PIG-A* gene located on the X chromosome (Dunn et al., 1999; Socié, et al., 2000).

There are three types of PNH cells, type I, type II and type III, all of which show various sensitivity levels to complement. Patients with PNH may present with AA-like symptoms (pancytopenia with hypoplastic marrow) or MDS-like

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symptoms (pancytopenia with cellular marrow), and cytogenetic analyses have not provided any diagnostic chromosomal patterns. PNH is associated with a high rate of thromboses, which may be fatal, and renal abnormalities (mainly due to hepatic thromboses). Other than bone marrow transplantation, which is rarely used to treat patients with PNH, there is no definitive treatment regimen. Complete remission is only achieved in a small number of patients (Lee, 1993).

Recent reports show that approximately 22-23% of patients with AA or MDS also suffer from PNH (Dunn, et al., 1999). Originally, it was thought that PNH was a late complication of AA and MDS, however more recent data show that cells deficient in GPI-anchored proteins (detected by flow cytometry) are present at the time of diagnosis with AA or MDS (Colby et al., 1996; Dunn, et al., 1999; Socié, et al., 2000; Tisdale, et al., 2000; Young, 2000). Cells deficient in GPI-anchored proteins were not detected in patients treated with antithymocyte globulin (ATG) for reasons other than AA or MDS (Dunn, et al., 1999). This suggests that there is no correlation between ATG therapy and the presence of cells lacking GPI-anchored proteins (Dunn, et al., 1999; Socié, et al., 2000). However, MDS patients who express cells without GPI-anchored proteins

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respond better to treatment with immunosuppressive therapy, and recent experiments by Dunn et al. showed that cells which are deficient in GPI-anchored proteins are less affected by an autoimmune attack (Dunn et al., 1999). This phenomenon may explain why the onset of PNH may be linked with the presence of a bone marrow failure disorder (AA or MDS) that is thought to be a result of an autoimmune attack. Since cells lacking GPI-anchored proteins show resistance to attack by the immune system, they may be preferentially selected for in the case of such an attack.

Myelodysplastic Syndromes (MDS)

The MDS are a group of clonal disorders that can occur de novo, or may occur following treatment for AA (secondary MDS). They are characterized by anemia that does not respond to treatment (Deiss, 1993). Secondary MDS has a characteristic pattern of evolution wherein hematopoiesis is restored (at least partially) after treatment for AA, followed by a subsequent decline and the onset of MDS or other disorders (see PNH section above) (De Planque et al., 1988; Socié et al., 2000). MDS is associated with an older age group, and is slightly more common in males (Barrett et al., 2000; Deiss, 1993). There are five categories of MDS as defined by the French, American, and British (FAB)

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group: refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-t), and chronic myelomonocytic leukemia (CMML) (Deiss, 1993). Of these subsets, patients diagnosed with RAEB or RAEB-t are more likely to undergo leukemic transformation. Unfortunately, classification is based on phenotypic markers alone, and the presentation of MDS can often be misdiagnosed as AA and vice versa (Barrett et al., 2000; Dunn et al., 1999; Socié et al., 2000). MDS often presents clinically with one or more cytopenias (anemia, pancytopenia, thrombocytopenia, or neutropenia) present at the time of diagnosis (Barrett et al., 2000; Deiss, 1993). In addition, cytogenetic abnormalities are commonly found at diagnosis, including 5q-, monosomy 7 and trisomy 8. A complex karyotype, or the presence of monosomy 7 is considered to be a poor prognosis (Barrett et al., 2000; Deiss, 1993; Socié et al., 2000). It is estimated that 10-25% of patients with MDS will progress to acute nonlymphocytic leukemia (ANLL). The development of MDS may be due to immunosuppressive therapy for a prior malignancy (therapy-related MDS (t-MDS)) (Deiss, 1993; Socié et al., 2000). In t-MDS, a greater percentage of patients have complex cytogenetics, do not respond well to

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treatment, and a larger percentage undergo transformation to ANLL (Deiss, 1993).

Many patients are asymptomatic, and chemotherapy is usually unsuccessful. It is hard to evaluate the effectiveness of various treatment regimens due to the lack of large randomized trials with patients of the same disease category (de novo versus secondary MDS, etc.). Remission is attained in approximately 50% of patients with MDS, however relapse is very common and occurs rapidly. Bone marrow transplant is an available option for some patients with an HLA-matched donor, but as with all bone marrow transplants, complications such as graft versus host disease (GVHD) may occur (Deiss, 1993).

Telomere shortening has been associated with progression to ANLL, an increase in cytogenetic abnormalities and a poor prognosis (Rosenfeld and List, 2000). Since telomeres are responsible for genomic stability, it is possible that the shortening of the telomeres, due to an increase in cell divisions (when the bone marrow is under stress) and complicated by advanced age (Jordan and Van Zant, 1998), may contribute to an increase in genomic instability and neoplastic progression (Marsh, 2000; Rosenfeld and List, 2000).

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Collectively, the standing hypothesis regarding the evolution of AA to MDS is as follows: 1) initial injury to a progenitor cell occurs, possibly due to toxin exposure, 2) increased apoptosis of marrow cells (onset of AA), 3) enhancement of telomere shortening resulting in genomic instability (development of early MDS), 4) a second insult to the cells, perhaps due to inactivation of a tumor suppressor gene, and lastly 5) progression to advanced MDS and ANLL (Barrett, et al., 2000; Rosenfeld and List, 2000).

Acute Nonlymphocytic Leukemia (ANLL)

ANLL is often referred to as acute myeloid leukemia (AML); however, ANLL is the more precise term and will therefore be used in this manuscript. ANLL is the culmination of disease progression that can start with the development AA followed by evolution to MDS and ending in ANLL. Environmental toxins, such as benzene, which contribute to the onset of AA and MDS also contribute to the onset of ANLL. In addition, ANLL may arise after chemotherapy, which damages the DNA. Chromosomal instability has been implicated in the development of ANLL, and cytogenetic abnormalities are common in most patients with ANLL (Greer et al., 1999; Greer and Kinney, 1993).

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ANLL is a clonal disorder that often presents with various cytopenias (neutropenia, thrombocytopenia) and the presence of blast cells (immature cells) in the bone marrow. Like MDS, the classification system for ANLL is poor and is based on phenotypic markers. ANLL has seven subcategories as defined by the FAB system: M1, M2 and M3 ANLL are mainly granulocytic, M4 ANLL shows >20% of the cells having monocytic origin, M5 ANLL shows >80% of the cells with a monocytic origin, M6 ANLL involves erythroid differentiation, and M7 ANLL is megakaryocytic (Greer et al., 1999; Greer and Kinney, 1993).

ANLL M2, M3 and M4 are associated with a good prognosis, and ANLL M5, M6 and M7 are associated with a poorer prognosis. Certain cytogenetic abnormalities are commonly seen in association with a particular subtype, such as t(8;21) with ANLL M2 and t(15;17) with ANLL M3. These translocations often involve known oncogenes and could result in their activation or the suppression of a tumor suppressor gene. Secondary ANLL is often associated with monosomy 7 or 5q-, which are considered to be indicators of a poor prognosis (Greer et al., 1999; Greer and Kinney, 1993). It is of interest to note that genes for five major cytokines are located in the 5q region (Deiss, 1993).

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As with other neoplasms, therapy involves chemotherapy or bone marrow transplantation. Chemotherapy involves a high dose of induction therapy, usually cytosine arabinoside combined with an anthracycline, followed by post-remission therapy. The cure rate using chemotherapy alone is between 10-30%, while the cure rate with bone marrow transplantation is 45-65%. Patients with preceding MDS often have lower response rates to chemotherapy, and involvement of the central nervous system indicates a poor prognosis. Unfortunately, 40-80% of all patients will relapse, and 10-20% of patients with ANLL do not respond to any treatment (Greer et al., 1999; Greer and Kinney, 1993).

Aplastic Anemia (AA)

Background

AA is a bone marrow failure disorder that can progress to pre-leukemic conditions (MDS, etc.) and leukemia (Barrett et al., 2000). AA is characterized by the lack of precursor cell components for most cell types (Marsh, 2000; Young, 1999). Essentially, bone marrow production ceases; there is a reduction in red marrow (hematopoiesis) and an increase in yellow marrow (inactive fat cells) (Peterson, 1995; Young, 1999; Young, 2000) (Figure 2). This condition is referred to as pancytopenia (Barrett et al., 2000; Lee,

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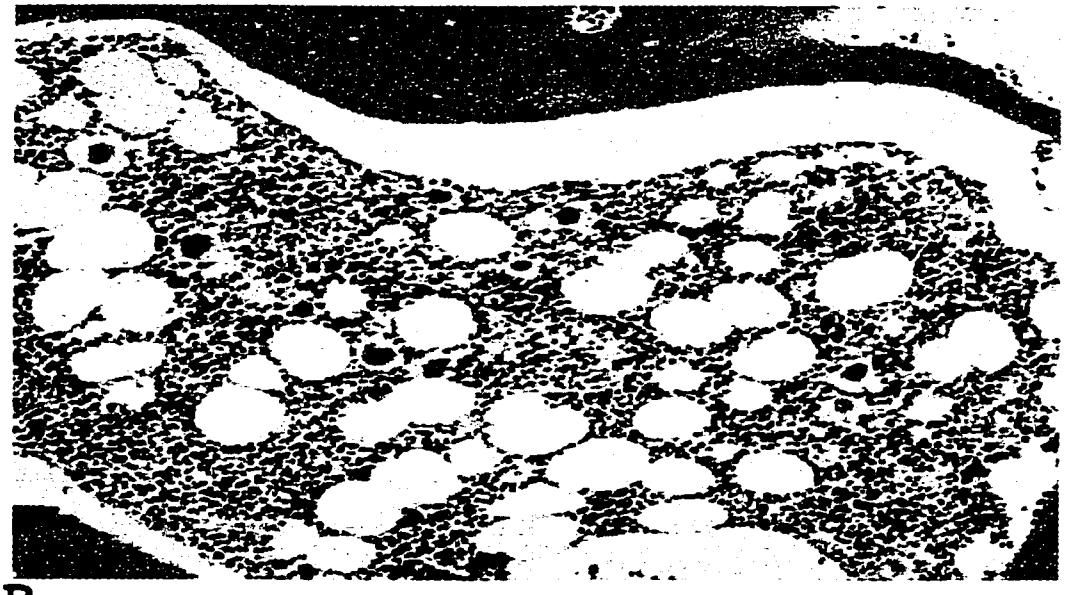
A



B

Figure 2. Hematopoiesis

(A) Normal bone marrow showing correct ratio between red and yellow marrow components. Photograph taken from http://www.vet.purdue.edu/vpb/clinpath/vpb555/lect1hematopoiesis/

(B) Bone marrow failure showing a decrease in red marrow and an increase in yellow marrow (Young, 1999)

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1999; On-line Medical Dictionary, 2001; Williams, 1993; Young, 2000). There are many different pathways that lead to the development of AA, causing difficulty in proper diagnosis and treatment for AA patients (Guinan, 1997). AA can occur from exposure to toxic chemicals such as benzene (via inhalation) Colby et al., 1996; Lee, 1999; Marsh and Geary, 1991; Young, 1999). Benzene can bind to DNA and RNA, creating adducts, which can degrade and contribute to DNA breakage (Shackelford et al., 1999). Radiation has also been linked to the development of AA through nuclear accidents or accidental exposures (Colby et al., 1996; Guinan, 1997; Lee, 1999; Marsh and Geary, 1991; Young, 2000). In addition, there are a wide variety of drugs that can induce AA, such as chloramphenicol (Colby et al., 1996; Guinan, 1997; Lee, 1999; Young, 1999; Young, 2000). Since there are no firm associations between drug dosages and the amount of time a drug was administered, it is hard to determine which drugs have a detrimental effect (Lee, 1999). Recently, AA has been associated with hepatitis patients, although a specific connection remains unidentified (Guinan, 1997; Lee, 1999; Young, 2000). AA may also be of familial origin, as in the case of Fanconi's anemia (FA), an autosomal recessive disorder characterized by various abnormalities of the skeletal and urogenital

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systems, hyperpigmentation and pancytopenia (Marsh and Geary, 1991; Russo and Zwerdling, 1992; Smith and Cox, 1999; Thurston et al., 1999). However, the vast majority of AA cases are idiopathic (due to unknown causes) (Colby et al., 1996; Lee, 1999; Online Medical Dictionary, 2001; Peterson, 1995; Storb, 1997; Young, 2000).

Patients with AA present with one or more of the following clinical characteristics: a low number of red blood cells (anemia), a low number of white blood cells (leukopenia) and/or a low number of platelets (thrombocytopenia) (Lee, 1999; Online Medical Dictionary, 2001). Due to the wide variety of abnormalities with symptoms and clinical findings similar to AA (PNH, MDS), diagnosis is often difficult (Lee, 1999; Young, 1999). Occasionally, cytogenetic abnormalities are present, which indicates a poor prognosis and a greater potential for disease progression to MDS or ANLL (Barrett et al., 2000; Kaito et al., 1998; La Starza et al., 1998; Ohara et al., 1997; Thurston et al., 1999).

The majority of patients with AA have a normal karyotype analysis (Barrett et al., 2000). In patients with severe aplastic anemia (SAA) (criteria established by the International Aplastic Anemia Study Group), cytogenetic abnormalities usually include monosomy 7, trisomy 8 and/or

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trisomy 21 (Barrett et al., 2000; Colby, et al., 1996; La Starza et al., 1998; Ohara et al., 1997; Russo and Zwerdling, 1992; Thurston et al., 1999). As with MDS, the presence of monosomy 7 is considered a poor prognosis (Thurston et al., 1999). The degree of severity of AA is diagnosed using the standards set forth by the International Agranulocytosis and Aplastic Anemia Study Group (Colby et al., 1996; Kaito et al., 1998; Lee, 1999).

It is estimated that 10% of patients with AA will develop PNH and 5-15% of patients with AA will progress to MDS or ANLL (Kaito et al., 1998; Ohara et al., 1997; Socié et al., 2000; Tisdale, et al., 2000; Young, 1999). In those patients who progress to MDS, chromosomal abnormalities are frequently seen, and assist in the diagnosis of MDS (Barrett et al., 2000; Kaito et al., 1998; Ohara et al., 1997; Socié et al., 1993; Young, 1999). Recent studies have linked the development of cytogenetic abnormalities such as monosomy 7 and trisomy 21 to previous treatment with G-CSF (Kaito et al., 1998; Ohara et al., 1997; Young, 1999). This link has been noted in both children (Ohara et al., 1997) and adults (Kaito et al., 1998). G-CSF stimulates myelopoiesis and can assist in combating infection following immunosuppressive therapy (Frickhofen and Rosenfeld, 2000; Marsh, 2000; Sachs, 1996; Young, 1999).

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However, it is important that treatment with any cytokine be appropriately timed to ensure the destruction of all malignant cells (Sachs, 1996). It is theoretically possible that the treatment of patients, in the Ohara and Kaito studies, with both immunosuppressive therapy and G-CSF simultaneously may have resulted in protection of malignant cells from apoptosis followed by an expansion of the malignant cells. The common factor in all patients who exhibited complications, such as MDS or PNH, was treatment with G-CSF for over one year (Kaito et al., 1998; Marsh, 2000).

A recent study, of a patient with FA, using fluorescence *in situ* hybridization (FISH), showed a higher percentage of cells with monosomy 7 than was recorded using conventional metaphase karyotype analysis. In addition, retrospective analysis indicated an increase in monosomy 7 levels over time (Thurston, et al., 1999). This and other studies show that FISH can detect chromosome abnormalities that are undetected by a karyotype analysis (Thurston et al., 1999; Socié et al., 2000; Young, 1999; Young, 2000). Such analysis is particularly useful in patients with AA, since routine karyotype analysis can be difficult because there is a lack of dividing cells (Barrett et al., 2000; De

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Planque et al., 1988; Socié et al., 2000; Tichelli et al., 1988).

Numerous recent reports support the hypothesis that acquired AA is an autoimmune disorder that is caused by attack on the hematopoietic system by lymphocytes (T-cells) (Colby et al., 1996; Tisdale, et al., 2000; Young, 1999; Young, 2000). Lymphocytes are recruited via cytokine signals (INF, IL2 and TNF), which leads to the activation of apoptosis via the Fas/Fas ligand pathway (Tisdale, et al., 2000; Young, 1999). INF-γ and TNF-α levels are increased in patients with SAA, which can cause an increase in Fas-mediated apoptosis (Colby et al., 1996; Young, 2000). This autoimmune destruction may select for cells which are unaffected by autoimmune attacks such as PNH clones (see previous PNH section) or cells that are resistant to apoptosis (MDS), causing clonal expansion of those cells and the manifestation of the clinical disorders (Young, 1999; Young, 2000) (Figure 3).

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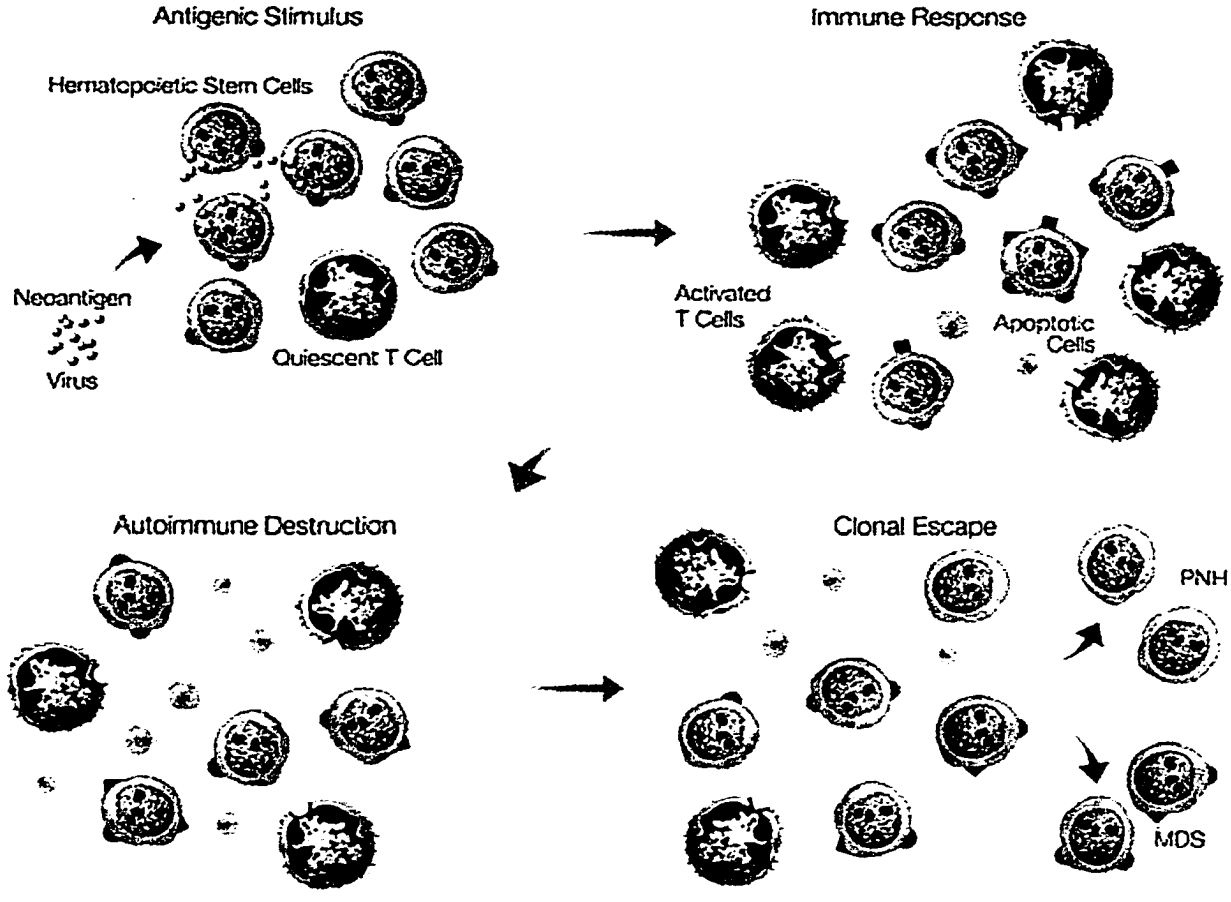

Figure 3. Hypothetical Pathway of Autoimmune Destruction

Stem cell insult occurs from drug exposure etc. (a viral infection is shown here), which causes a change in the production of the cell proteins and cell surface markers. T cells are activated by a normal immune response to "foreign" cells and apoptosis occurs. Emergence of resistant clones results in clinical manifestations (Young, 1999).

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Epidemiology

AA is primarily a disease of the young, with an average age of twenty-five (Young, 2000). In the United States and Europe, the incidence of AA is approximately 2 per million (Storb, 1997; Young, 1999; Young, 2000), while rates in the Far East are as high as 11 per million (Storb, 1997). AA is more common in developing nations perhaps owing to the lack of industrial standards and a higher rate of exposure to toxins such as benzene (Lee, 1999). AA is slightly more common in males (Barrett et al., 2000). However, in a study by Kaito et al. (1998), looking at the rate of transformation from AA to MDS, no statistical correlation was found with respect to the age of the patient, the sex of the patient, or the severity of the disease.

Treatment

Bone marrow transplant is the treatment of choice, except in cases where the age of the patient or lack of a suitable donor prohibits transplantation (Colby et al., 1996; Guinan, 1997; Lee, 1999; Young 2000). Survival rates of up to 90% are achieved in young patients who undergo bone marrow transplantation prior to the increase of GVHD, which is found in patients over 30 years of age (Young, 2000). Whole body irradiation has decreased the rate of GVHD;

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however, the consequences of total irradiation can lead to future complications or death (Lee, 1999). Patients with no history of blood transfusions prior to bone marrow transplantation have a better chance of survival and remission (Colby et al., 1996; Guinan, 1997; Lee, 1999). Transfusions can increase the chance of graft rejection by causing an increased sensitivity to histocompatability antigens (Colby et al., 1996). The overall survival rate of patients undergoing bone marrow transplantation has continued to improve due to advances in technology (Guinan, 1997; Lee, 1999).

In cases where bone marrow transplant is not an option, immunosuppressive therapy is the second choice (Frickhofen and Rosenfeld, 2000; Guinan, 1997; Lee, 1999; Young, 1999; Young, 2000). Treatment usually consists of ATG alone or in combination with cylosporin A (CsA) (Colby et al., 1996; Guinan, 1997; Lee, 1999; Young, 1999; Young, 2000). ATG works by inhibiting T cell mediated immune response, although the exact mechanism is unknown (Colby et al., 1996; Frickhofen and Rosenfeld, 2000; Young, 2000). CsA inhibits the transcription of several cytokines, blocking an immune response (Frickhofen and Rosenfeld, 2000). The rate of survival in patients who respond to immunosuppressive therapy can reach as high as 90% (Young,

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2000). The responsiveness of a patient is associated with the severity of the disease (Colby, et al., 1996; Guinan, 1997; Lee, 1999). G-CSF has been administered to some patients with AA, however treatment with G-CSF alone cannot induce remission (Young, 2000). G-CSF stimulates the myelopoiesis, and can aid in the effectiveness of other immunosuppressive drugs (Frickhofen and Rosenfeld, 2000; Guinan, 1997; Marsh, 2000; Young, 1999; Young, 2000). Unfortunately, immunosuppressive treatment has been associated with an increased risk of developing a hematological disorder, such as PNH, MDS or ANLL (Frickhofen and Rosenfeld, 2000; Lee, 1999; Marsh, 2000; Tisdale, et al., 2000; Young, 2000). Research is ongoing to establish a better therapy regimen that increases overall survival and remission rates while decreasing the risk of developing future complications (Guinan, 1997; Lee, 1999).

Alternate therapies to ATG and CsA are being investigated such as cyclophosphamide, a potent immunosuppressive drug (Frickhofen and Rosenfeld, 2000; Tisdale, et al., 2000; Young, 1999; Young, 2000). Preliminary data shows that patients treated with cyclophosphamide do not develop clonal disorders (PNH or MDS) and do not relapse (Tisdale, et al., 2000; Young, 1999).

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In addition to immunosuppressive therapy, supportive care, such as regular blood transfusions, is part of the treatment regimen (Lee, 1999; Young, 2000). However, caution should be used when the patient is eligible for a bone marrow transplant, since patients who have not had any prior blood transfusions have a greater successful response rate to bone marrow transplantation (Lee, 1999). Another key to successful treatment that is often overlooked, is prevention of infection, which can only compound problems within an already reduced immune system (Lee, 1999).

CELL CYCLE

General Overview

By definition, the cell cycle is "the sequence of events between mitotic divisions" (Online Medical Dictionary, 2001). The end result from one round of cell division is two genetically identical daughter cells (Johnson and Walker, 1999). The cell cycle includes interphase (comprised of G1, S and G2) and mitosis (Figure 4). Cells that are quiescent are in G0, a non-dividing cell resting state (Jacks and Weinberg, 1998; Johnson and Walker, 1999; Shackelford et al., 1999).

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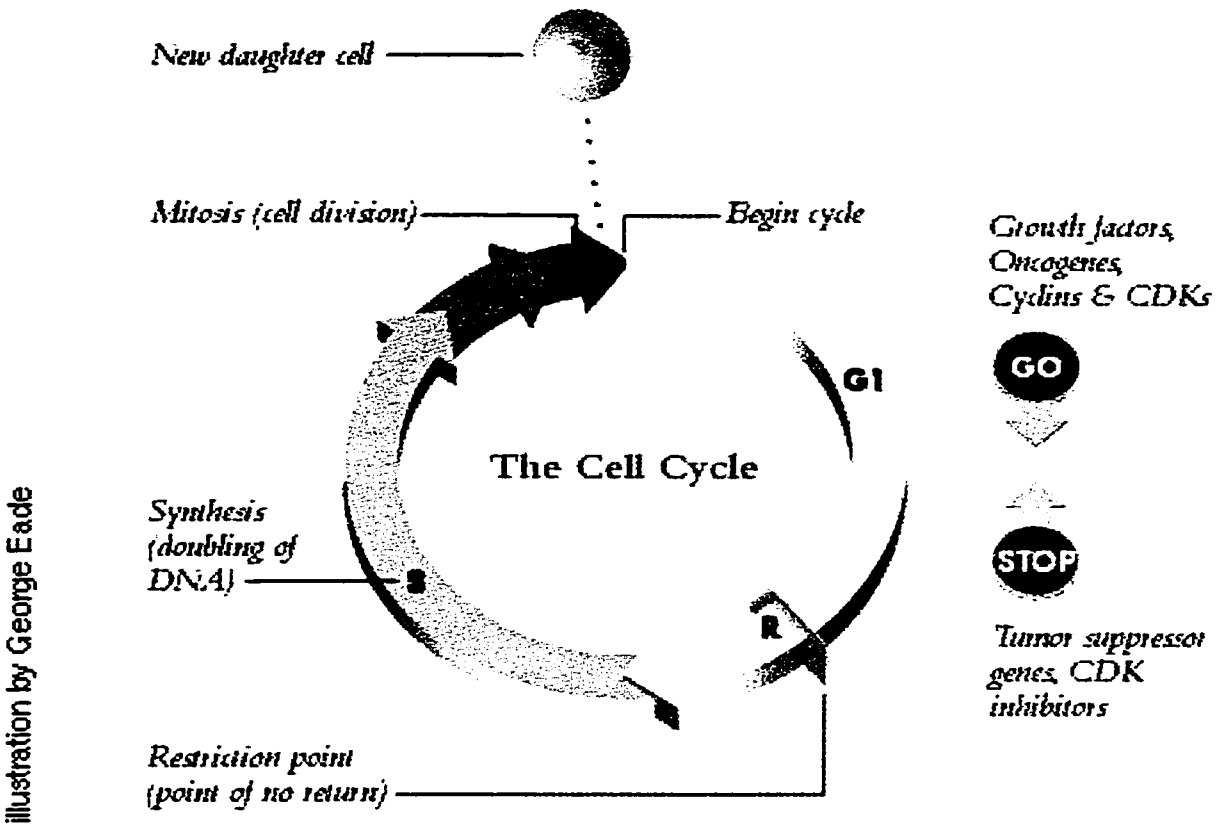

Figure 4. The Cell Cycle

Photograph taken from
http://www.geocities.com/CollegePark/Lab/1580/cycle.html

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During G1, the cell synthesizes RNA and proteins in preparation for DNA replication (Hagan et al., 1999; Johnson and Walker, 1999). G1 is the longest stage (in animals), comprising nearly half of the total cell cycle time (Shackelford et al., 1999). During the S phase, the DNA is replicated, and upon completion, the cell now contains two copies of the same DNA (Hagan et al., 1999; Johnson and Walker, 1999). S phase comprises almost one quarter of the entire cell cycle time (Shackelford et al., 1999). During G2, the cell prepares to enter mitosis (Hagan et al., 1999; Johnson and Walker, 1999). G2 phase comprises less than one quarter of the total cell cycle time. G2 is followed by mitosis, which is the shortest part of the cell cycle. The cell cycle repeats every 18-24 hours (Shackelford et al., 1999).

Mitosis is broken down into four substages: prophase, metaphase, anaphase and telophase. During prophase, the mitotic spindle begins to form in the cytoplasm. The chromatin condenses, forming the X-shaped chromosomes, and the centrosomes, comprised of two centrioles, begin to move apart. In preparation for metaphase, the nuclear envelope disintegrates, and the centrosomes localize to the spindle poles on opposite sides of the cell. The spindle fibers, comprised of microtubules, attach to the kinetochores,

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which are located in the centromeric region of each chromosome (Willard, 2000). In metaphase, the chromosomes attach to the spindle apparatus as they begin to align along the metaphase plate in between the two spindle poles. During anaphase, the sister chromatids divide and move toward opposite poles with help from the spindle apparatus attached to the kinetochore region. During the final phase of mitosis, telophase, the nuclear envelopes begin to reform, with one envelope surrounding each set of chromosomes. The chromatin begins to uncoil, and the cytoplasm divides (cytokinesis) (Campbell, 1993).

Mitotic Cell Cycle Checkpoint Mechanisms

Many cell cycle controls (checkpoints) are built into the cell cycle to ensure correct cell division (Johnson and Walker, 1999; Shackelford et al., 1999). Cell cycle checkpoints are mediated by the activity of cyclin/cdk (cyclin-dependent kinase) complexes (Hagan et al., 1999; Shackelford et al., 1999). During interphase, there are two main checkpoints. The first checkpoint is a restriction point located prior to the S phase (Hartwell and Kastan, 1994; Johnson and Walker, 1999; Shackelford et al., 1999) (Figure 4 and Figure 5). Here, the cell must decide whether to continue in the cell cycle or abort and

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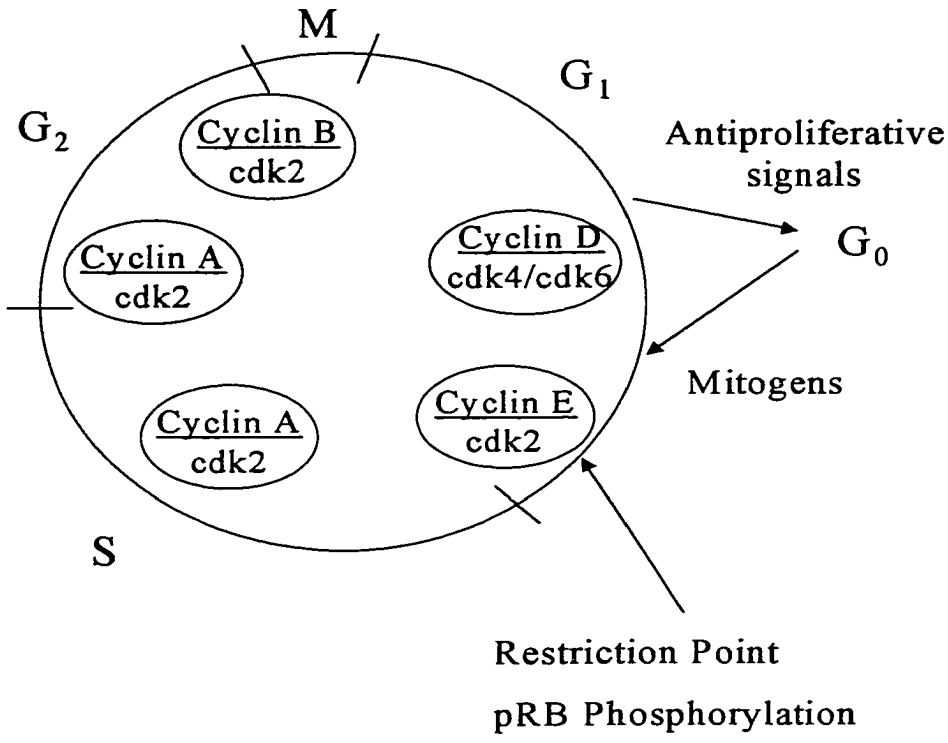

Figure 5. Cyclin/cdk Complexes Within the Cell Cycle

Pictorial representation of the Cyclin/cdk protein complexes and their locations within the cell cycle control (Shackelford et al., 1999).

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enter the G0 quiescent stage (Johnson and Walker, 1999). Once the cell has decided to continue with the cell cycle, there is no turning back (Johnson and Walker, 1999; Shackelford et al., 1999). The G1 checkpoint ensures that damaged DNA is not replicated (Hartwell and Kastan, 1994). The second checkpoint occurs in late G2, prior to entering mitosis (Johnson and Walker, 1999; Shackelford et al., 1999). This checkpoint is controlled by the levels of maturation (or M phase) promoting factor (MPF), and ensures that DNA replication is complete prior to the initiation of mitosis (Hartwell and Kastan, 1994; Shackelford et al., 1999).

D-type cyclins induce the division of G0 cells. The levels of D-type cyclins remain constant throughout the cell cycle (Johnson and Walker, 1999). D-type cyclin kinases phosphorylate the retinoblastoma tumor suppressor protein (Rb), which is involved in the restriction point during G1 (Figure 5) (Bartek et al., 1999; Johnson and Walker, 1999; Shackelford et al., 1999). After phosphorylation, the Rb protein dissociates from E2F, a transcription factor that regulates the expression of cyclin E. The formation of a cyclin E/cdk2 complex is required for the transition into S phase (Figure 5). Cyclin A negatively regulates E2F (Johnson and Walker,

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1999; Shackelford et al., 1999). Entry into mitosis is controlled by MPF, which is controlled by the level of cyclin B (Hagan et al., 1999; Shackelford et al., 1999). Cyclin B binds to $p34^{cdc2}$ *(cell division cycle 2)*, a cdk that is part of the MPF (Figure 5) (Shackelford et al., 1999). Upon binding to cyclin B, and after phosphorylation, $p34^{cdc2}$ becomes active. When $p34^{cdc2}$ is active, it will activate the MPF, phosphorylate many cell cycle components, and initiate the formation of the mitotic spindle (Hagan et al., 1999; Shackelford et al., 1999). During mitosis, cyclins A, B1 and B2 associate with cdk1, and are responsible for phosphorylation of a wide variety of targets including components of the mitotic spindle (Johnson and Walker, 1999; Shackelford et al., 1999). Mitosis is completed when the destruction of cyclin B inhibits MPF function (Hagan et al., 1999). Once the cell has completed mitosis, the process begins anew with cyclin building up until it reaches the threshold level required to initiate cell division (Hagan et al., 1999; Johnson and Walker, 1999).

Cyclin-dependent kinase inhibitors (CKI) regulate the cyclin/cdk complexes (Johnson and Walker, 1999). One member of this family is p21, which is activated by p53, and (when active) is responsible for the inhibition of the cell cycle. Activation of p21 halts DNA replication to

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allow for repair of damaged DNA (Johnson and Walker, 1999; Shackelford et al., 1999). Therefore, certain cancers that have altered p53 function may be unable to activate p21, which would allow damaged DNA to undergo replication. Mutations in p53 are common in cancer, which leads to an increase in genomic instability manifested by a high level of chromosomal abnormalities (Johnson and Walker, 1999). Another CKI, p16, is known to inhibit Rb phosphorylation (Johnson and Walker, 1999; Shackelford et al., 1999). When Rb phosphorylation is inhibited, E2F is not activated and the cell cannot undergo DNA replication.

During metaphase, the chromosomes align along the metaphase plate prior to their separation during anaphase. Separation of chromosomes is assisted by microtubules in the mitotic spindle that are attached to one of the two microtubule organizing centers (MTOCs) located at the spindle poles. Movement of the chromosomes is accomplished by microtubule motor proteins (Hagan et al., 1999). The mitotic spindle checkpoint ensures that all chromosomes are aligned properly and attached to the spindle apparatus before division occurs (Davenport et al., 1999; Elledge, 1998; Hwang et al., 1998; Imai et al., 1999; Nicklas, 1997; Paulovich et al., 1997). The mitotic spindle checkpoint is comprised of genes from the *MAD* (mitotic arrest deficient)

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and *BUB* (budding uninhibited by benzimidazole) families and is discussed in further detail below (Shackelford et al., 1999). A defect in the mitotic spindle checkpoint would allow premature anaphase and may lead to uneven chromosome division.

BUB and _MAD_ Gene Families

In the yeast *Saccharomyces cerevisiae*, there are seven genes identified (to date) which are required for the spindle assembly checkpoint. These include *BUB* genes *1*, *2* and *3*, *MAD* genes *1*, *2* and *3*, and *MPS1*, (monopolar spindle 1) a protein kinase (Basu, et al., 1999; Bernard et al., 1998; Hardwick, et al., 1996; Jin et al., 1998; Pangilinan et al., 1997). Mammalian homologues of BUB1, BUB3, MAD1, MAD2 and MAD3 (which is occasionally referred to as BUBR1, due to similarities to both the *BUB* and *MAD* gene families) localize during prometaphase to the kinetochore region of chromosomes that are not attached to the spindle apparatus, and disappear once stable attachment is achieved during metaphase (Elledge, 1998; Fraschini et al., 1999; Hardwick et al., 2000; Li and Benezra, 1996; Li, 1999). However, BUB2 localizes to the spindle poles, indicating a potentially different function from BUB1, BUB3, MAD1 and MAD2 (Fraschini et al., 1999; Hardwick et al., 2000; Li,

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1999). Many hypothesize that BUB1, BUB3 and MAD1-3 proteins all form a complex that targets unattached kinetochores, comprising the mitotic spindle checkpoint (Fraschini et al., 1999; Li, 1999; Seeley et al., 1999). It is also hypothesized that BUB2 assists in the regulation of cytokinesis (Fraschini et al., 1999; Li, 1999). Evidence supporting this theory is found in fission yeast where cdc16, the fission yeast homologue of BUB2, negatively regulates cytokinesis, and is localized to the spindle poles (Fraschini et al., 1999; Li, 1999). The kinetochore-binding domain CENP-E has also been implicated in the mitotic spindle checkpoint (Chan et al., 1998). Like the other checkpoint proteins, CENP-E localizes to the kinetochore (Chan et al., 1998). CENP-E is a motor-kinase and is thought to contribute in the shortening of the spindle apparatus during metaphase (Chan et al., 1998).

There is evidence that BUB1, BUB3 and MAD1-3 proteins all interact (Figure 6). BUB1 undergoes autophosphorylation and also phosphorylates BUB3 (Farr and Hoyt, 1998; Hardwick, 1998; Martinez-Exposito et al., 1999; Roberts et al., 1994; Taylor and McKeon, 1997). In addition, the binding of BUB3 to BUB1 is necessary for kinetochore localization of BUB1 (Fraschini et al., 1999; Seeley et al., 1999; Taylor et al., 1998). MAD3 (BUBR1) binds to

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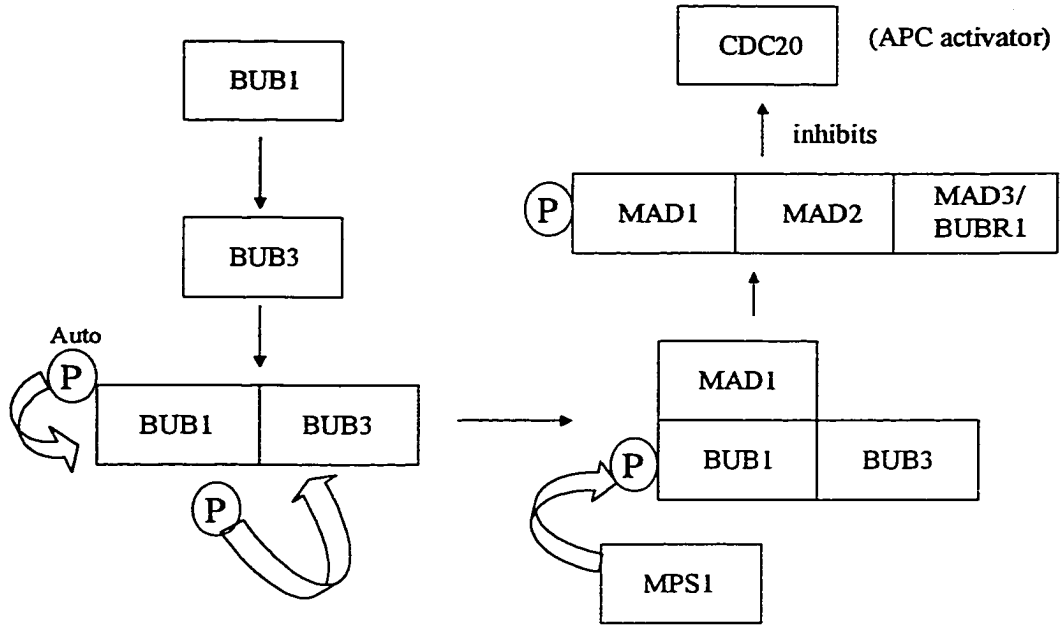

Figure 6. Hypothesized Interaction of the BUB/MAD Proteins, and Their Possible Role in the Regulation of the Mitotic Spindle Checkpoint.

BUB1 binds to BUB3, which is necessary for kinetochore localization of BUB1. BUB3 is phosphorylated by BUB1 and BUB1 is autophosphorylated. The BUB1/BUB3 complex binds to MAD1, and MAD1 is phosphorylated by MPS1. From there, it is hypothesized that MAD2 and MAD3 (BUBR1) are added to the complex. By an unknown mechanism, the newly formed complex inhibits CDC20 which prevents activation of the APC. By inhibiting the activation of the APC, anaphase is blocked.

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BUB3, (Hardwick et al., 2000; Taylor et al., 1998) and MAD1 binds to BUB1 (Seeley et al., 1999). MAD 1-3 interact with each other and with the anaphase promoting complex (APC) in yeast, and MAD2 is able to inactivate CDC20 and stabilize Pds1 (in *S. cerevisiae*) and cyclin B. This blocks the onset of anaphase by preventing the destruction of Pds1 and cyclin B (Elledge, 1998; Hardwick et al., 2000; Hwang et al., 1998; Li and Benezra, 1996; Taylor et al., 1998). MAD1 binds to MAD2, and MAD1 is phosphorylated by MPS1 (Bernard et al., 1998; Farr and Hoyt, 1998; Fraschini et al., 1999; Taylor and McKeon, 1997; Taylor et al., 1998). CENP-E has been shown to colocalize and bind to MAD3 (BUBR1) at the kinetochore region (Chan et al., 1998; Chan et al., 1999).

hBUB1

Characteristics

The human BUB1 protein is a 1,021 amino acid (aa), 188-kDa protein which contains three main functional domains: CD1, which is highly conserved and directs kinetochore localization, NLS, a nuclear localization signal sequence and CD2, a highly conserved carboxy-terminal serine-threonine-like protein kinase domain (Cahill et al., 1999;

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Pangilinan et al., 1997; Roberts et al., 1994). Both kinetochore localization (the BUB1 protein must be near the kinetochore region to facilitate in the binding of the mitotic spindle to the kinetochore) and nuclear localization (the BUB1 protein must be within the nucleus of the cell to be able to localize to the kinetochore) of *hBUB1* are essential to the function of hBUB1. hBUB1 is a protein kinase with 25 exons located on chromosome 2q12-q14 (Cahill et al., 1999; Pangilinan et al., 1997; Roberts et al., 1994). Exons 2-5 (codons 21-152) encode for the CD1 region, while exons 8 and 9 (codons 207-319) encode for the NLS region, and exons 20-25 (codons 732-1043) encode for the CD2 region. A hBUB3 binding site is located between CD1 and CD2 in the presumptive NLS area (Cahill et al, 1998; Cahill et al., 1999). Cahill et al. found that two of 19 colorectal cancer cell lines had mutations within *hBUB1*, and also showed high levels of aneuploidy (Cahill et al., 1998).

BUB1 homologues have also been found in a variety of organisms, including mice (Taylor and McKeon, 1997), *Drosophila* (Basu et al., 1999), fission yeast *Schizosaccharomyces pombe* (Bernard et al., 1998) and *Aspergillus nidulans* (Efimov and Morris, 1998). Bernard et al. showed that in *S. pombe*, BUB1 was also localized to the

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kinetochore region upon activation of the spindle checkpoint. In addition, loss of the *BUB1* gene in *S. pombe* instigated chromosomal segregation defects, from failure of sister chromatid segregation, producing a higher aneuploidy rate, and an increase in lagging chromosomes in late anaphase (Bernard et al., 1998). Taylor and McKeon showed that when the function of the murine homologue of BUB1 is abrogated (a suppressed mitotic spindle checkpoint), cells successfully avoid apoptosis and continue in the cell cycle (Taylor and McKeon, 1997). Basu et al. found that in *Drosophila*, severe mutations of *BUB1* (missgregation and fragmentation) were responsible for elevated levels of chromosome abnormalities, and ultimately death of the *Drosophila* larvae (Basu et al., 1999). In contrast to Taylor and McKeon's findings (above, Taylor and McKeon, 1997), severe mutations of *BUB1* in *Drosophila* induced a higher apoptotic state (Basu et al., 1999). The study of *BUB1* in *Drosophila* is the first study to assess *BUB1* in a multicellular organism (Basu et al., 1999).

GENOMIC INSTABILITY AND CANCER

The hallmark of cancer is an uncontrolled cell cycle (Bartek et al., 1999; Hartwell and Kastan, 1994; Johnson

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and Walker, 1999). The accumulation of mutations may lead to cancer (Imai et al., 1999: Ohshima et al., 2000; Orr-Weaver and Weinberg, 1998). Many hypothesize that genetic instability leads to an increase in the mutation rate and the onset of cancer (Boland and Ricciardiello, 1999; Cahill et al., 1998; Hartwell and Kastan, 1994; Imai et al., 1999; Jaffrey et al., 2000; Lengauer et al., 1997; Myrie et al., 2000; Ohshima et al., 2000). There are two forms of genetic instability: chromosomal instability (CIN) and microsatellite instability (MIN) (Cahill et al., 1999; Imai et al., 1999; Jaffrey et al., 2000).

Chromosomal Instability

CIN is characterized by malsegregation of chromosomes and presents as aneuploidy at the chromosomal level (Bernard et al., 1998; Cahill et al., 1998; Cahill et al., 1999; Imai et al, 1999; Jaffrey et al., 2000; Lengauer, et al., 1997; Myrie et al., 2000; Orr-Weaver and Weinberg, 1998). Aneuploidy and other chromosomal aberrations are frequently associated with a variety of cancers and tumors (Boland and Ricciardiello, 1999; Davenport et al., 1999; Hartwell and Kastan, 1994; Imai et al., 1999; Lengauer et al., 1997; Myrie et al., 2000; Yamaguchi et al., 1999). Theoretically, aneuploidy may occur as a direct result of

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an aberrant mitotic spindle checkpoint (Bernard et al., 1998; Cahill et al., 1998; Cahill et al., 1999; Jaffrey et al., 2000; Ohshima et al., 2000; Orr-Weaver and Weinberg, 1998). Cells that proceed to anaphase before all chromosomes are aligned along the metaphase plate may result in a gain or loss of chromosomes. Monosomy 7 and other abnormalities have been observed in patients with AA, MDS and ANLL (Barrett et al., 2000; De Planque et al., 1988; Kaito et al., 1998; Ohara et al., 1997; Socié et al., 2000), and monosomy 7 is associated with a poor prognosis (Greer, et al., 1999).

Microsatellite Instability

MIN is characterized by mutations in mismatch repair genes, and results in mutations at the nucleotide level (Boland and Ricciardiello, 1999; Cahill et al., 1998; Cahill et al., 1999; Lengauer et al., 1997; Orr-Weaver and Weinberg, 1998). Tumors with MIN usually contain the correct number of chromosomes and do not exhibit aneuploidy (Orr-Weaver and Weinberg, 1998). However, nucleotide mutations may occur in oncogenes and tumor suppressor genes, leading to the inactivation of tumor suppressor genes or the activation of oncogenes (Cahill et al., 1999).

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HYPOTEHSIS AND RESEARCH AIMS

In this study, I hypothesized that mutations or base pair changes within the mitotic spindle checkpoint gene *hBUB1* directly correlated with the presence of aneuploidy in patients with bone marrow failure disorders. To test this hypothesis, two specific aims were addressed.

Recent experiments have shown that the emergence of abnormal clones in patients with AA coincides with a transformation to MDS (De Planque et al., 1988; Socié et al., 2000). In addition, as the disease progresses, more cytogenetic abnormalities occur (Socié et al., 2000). At present, karyotype analysis is used to evaluate the cytogenetic makeup of a patient. However, in patients with AA, this is often hard to accomplish because of the low number of metaphase cells present (Barrett et al., 2000; De Planque et al., 1988; Socié et al., 2000; Tichelli et al., 1988). Some researchers have shown that cytogenetic analysis by FISH is more sensitive than karyotype analysis (Thurston et al., 1999; Socié et al., 2000; Young, 1999; Young, 2000). In addition, FISH can be performed on patients with AA, since the procedure is primarily performed on interphase nuclei and does not rely on the existance of metaphase cells. Perhaps FISH analysis can

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detect early clonal abnormalities normally missed with karyotype analysis, and provide a "warning sign", indicating a potential transformation to MDS. Early clonal changes have been detected by karyotype in patients prior to transformation to MDS or ANLL (Marsh and Geary, 1991; Socié et al., 2000). In addition, a Seattle study showed that a greater percentage of AA patients with an abnormal karyotype underwent transformation to MDS or ANLL, while very few AA patients with a normal karyotype progressed to MDS or ANLL (Socié et al., 2000). Marsh and Geary have concluded that "an acquired clonal cytogenetic marker in a patient with otherwise typical AA represents a pre-leukaemic state" (Marsh and Geary, 1991). **Therefore, my first specific aim was to compare conventional karyotype analyses with FISH analyses in samples from patients with AA.** Initial cytogenetic testing using FISH analysis may complement a karyotype analysis, and could assist in a more complete and earlier diagnosis and guide correct preventative treatment in patients with AA. Patients with MDS and ANLL commonly present with either monosomy 7 or trisomy 8, of which, monosomy 7 is considered a poor prognosis (Socié et al., 2000). Therefore, FISH was performed for chromosomes 7 and 8.

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Secondly, aneuploidy is common in many cancers, and may be caused by a variety of things, such as structural defects in centrosomes or the mitotic apparatus, or defects in cell cycle control (Bartek et al., 1999; Pihan et al., 1998; Saunders et al., 2000). Some cancerous cell lines contained abnormal centrosomes and showed high levels of CIN and aneuploidy that were not seen in normal cell lines (Pihan et al., 1998). Other cancerous cell lines were shown to have multipolar spindles, perhaps due to a defect in the MTOCs (Saunders et al., 2000).

Theoretically, aneuploidy may also be caused from an aberrant mitotic spindle checkpoint. When the mitotic spindle checkpoint is viewed as a complex, one can assume that dysfunction in any of the components may compromise the function of the checkpoint. *hBUB1* is the most characterized of the mitotic spindle checkpoint genes, and was mutated within two colorectal cancer cell lines (Cahill et al., 1998). **Therefore, as my second specific aim, I chose to look for the presence of mutations or base pair changes within *hBUB1* in samples from patients with AA.** Since some mutations in *hBUB1* have a dominant negative effect (Cahill et al., 1998), any mutation that alters the function of BUB1 could potentially alter the mitotic spindle checkpoint. Should this be the case, one might

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predict to find a high level of aneuploidy in patients having a mutation in the *hBUB1* gene. Therefore, the same subset of patients underwent both FISH analysis and sequencing of the *hBUB1* gene functional domains shown to be required for kinetochore and nuclear localization (CD1 and NLS) (Cahill et al., 1999; Pangilinan et al., 1997; Roberts et al., 1994).

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CHAPTER II

MATERIALS AND METHODS

PATIENT SAMPLES

The National Institutes of Health, Heart, Lung and Blood Division in Bethesda, Maryland kindly provided patient samples consisting of whole bone marrow. The institutional review board authorized the use of human samples (IRB# 1303000246E)

SOLUTIONS

Below is a listing of solutions used in this study and their corresponding formulas:

- 2X SSC: Combined 100ml 20X SSC, pH 7.0 with 900ml water.
- 20X SSC, pH 7.0; 3M sodium chloride, 0.3M sodium citrate: Combined 175.3g sodium chloride (Sigma) and 88.2g sodium citrate (Sigma) in 800ml water. The pH was adjusted to 7.0 and water was added to a final volume of 1L.

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- 4X SSC/0.05% Tween 20, pH 7.0: Combined 200ml 20X SSC, pH 7.0 with 0.5ml Tween 20 (Fisher) and added water to a final volume of 1L.
- 4X SSC/0.05% Tween 20/0.05% blocker: Combined 200ml 20X SSC, pH 7.0 with 0.5ml Tween 20 and 0.5g blocker (Boehringer Mannheim). Water was added to a final volume of 1L.
- 50% formamide/2X SSC, pH 7.0: Combined 50ml formamide (Fisher), 10ml 20X SSC, pH 7.0 and 40ml water to a final volume of 100ml.
- 60% formamde/2X SSC/10% dextran sulfate: Combined 6ml formamide, 1ml 20X SSC, pH 7.0 and 1g dextran sulfate. Water was added to a final volume of 10ml. Solution was stored at -70°C until use.
- 70% formamide/2X SSC: Combined 70ml formamide, 10ml 20X SSC, pH 7.0 and 20ml water to a final volume of 100ml.
- DAPI, stock (10mg/ml): Dissolved 10mg DAPI (Sigma) in 1ml phosphate buffered saline (PBS).
- Ethidium Bromide, stock (10mg/ml): Dissolved 1g ethidium bromide (Sigma) in 100ml water. Solution was stored in the dark.
- Fixative: Combined 75ml methanol (Fisher) and 25ml glacial acetic acid (Fisher) for a final volume of 100ml.

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- Lennox broth (LB) agar plates: Combined 10g tryptone (Difco), 5g yeast extract (Difco), 5g sodium chloride, 1ml 1N sodium hydroxide (Fisher) and 15g agar (Difco) with water added to a final volume of 1L. Autoclaved. Cooled solution to 50°C and added the following antibiotics: 1ml ampicillin (50mg/ml) (Sigma), 1ml IPTG (100mM) (Sigma), and 1ml X-gal (20mg/ml) (Sigma). 15ml of the solution was aliquoted into a sterile 100mm petri dish and allowed to dry in a laminar flow hood for 30 minutes. Plates were stored at 4°C until use.
- LB media: Combined 10g tryptone, 5g yeast extract, 5g sodium chloride and 1ml 1N sodium hydroxide with water added to a final volume of 1L. Autoclaved. LB media was stored at 4°C until use.
- Phosphate buffered saline (PBS), 1x, pH 7.4; 0.14M sodium chloride, 0.003M potassium chloride, 0.01M sodium phosphate, dibasic, 0.002M potassium phosphate, monobasic: Combined 8g sodium chloride, 0.2g potassium chloride (Fisher), 1.44g sodium phosphate, dibasic (Fisher) and 0.24g potassium phosphate, monobasic (Fisher) in 800ml water. The pH was adjusted to 7.4 and water was added to a final volume of 1L. The solution was autoclaved.

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- Potassium chloride, 0.075M solution: Dissolved 2.8g potassium chloride in 500ml water.
- Tris-EDTA (TE) buffer, pH 7.4; 0.0007M Tris-Cl, 0.001M EDTA: Combined 0.112g Tris-Cl (Sigma) and 0.372g disodium thylenediamine tetraacetate (EDTA) (Fisher). Water was added to a final volume of 500ml.

HARVEST OF BONE MARROW CELLS

The bone marrow samples were harvested upon receipt using the following protocol: 0.075M KCl was warmed to 37°C while 0.5ml bone marrow sample was aliquoted into a labeled 15ml centrifuge tube. The cells were vortexed while adding warmed 0.075M KCl to a total volume of 5ml, followed by a 10 minute incubation in a 37°C water bath. While the cells were incubating, fresh fixative was prepared (see solutions). The cells were centrifuged at 1000 rpm for 5 minutes, and the supernatant was discarded to just above the white band of cells. The cells were vortexed while 5ml of fresh fixative was added *dropwise*, then the cells were re-centrifuged at 1000 rpm for 5 minutes. The addition of fixative followed by centrifugation was repeated twice. Again the cells were vortexed while 5ml of fresh fixative

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was added dropwise, and the cells were stored in fixative at -20°C until further use.

LABELING OF FISH DNA PROBES

In this study, a pre-mixed nick translation mix by Boehringer Mannheim was used according to the manufacturer's protocol. Briefly, dATP, dCTP, dGTP, dTTP, molecular biology grade (MBG) water (5 prime→3 prime), and a fluorescent label (see below) were combined to form the "5X fluorophore-labeling mix". Nick translation mix, template DNA and MBG water were added to the 5X fluorophore-labeling mix. The sample was incubated for 90 minutes at 15°C followed by a 10-minute incubation at 65°C. The samples were stored at -20°C until use.

Two-probe, two-color FISH was performed on chromosomes 7 and 8 simultaneously. Chromosome 7 was labeled with Biotin-16-dUTP (biotin-16-2'-deoxyuridine-5'-triphosphate) (Boehringer Mannheim) and detected with NeutrAvidin-fluorescein (Molecular Probes). Chromosome 8 was labeled with DIG-11-dUTP (digoxigenin-11-2'-deoxy-uridine-5'-triphosphate) (Boehringer Mannheim) and detected with anti-DIG rhodamine (Boehringer Mannheim).

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FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

Slides were prepared as follows: the fixed cells were centrifuged at 1000 rpm for 5 minutes, and the supernatant was discarded. Fresh fixative was prepared (see solutions), and while vortexing the cells, the fixative was added until a milky color was obtained. Slides were cleaned with ethanol (Fisher) and dried with a KimWipe (Kimberly Clark). Twenty microliters of the cell suspension was dropped onto each slide and allowed to air dry. The slides were stored at -70°C until use.

The FISH procedure was continued as follows: slides were first brought to room temperature. Then, the cells were incubated for 3 minutes in 70% formamide/2X SSC, pH 7.0 at 70-75°C to denature the DNA. The cells were washed in cold 50% ethanol, cold 70% ethanol and cold 95-100% ethanol, each for two minutes. While the cells were being washed, the probe mix was prepared by combining the following:

Labeled probe	1µl of each
Hybridization Buffer (60% formamide/ 2X SSC/10% dextran sulfate)	7µl
MBG water	1µl

The probe mix was denatured for 5 minutes at 80°C on a hot block and placed on ice. Slides were removed from the

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ethanol and air-dried. Ten microliters of the probe mix was placed on each slide; the slide was covered with a 22x22mm glass cover slip and sealed with rubber cement (Ross). Hybridization followed for 2-24 hours in a 37°C incubator.

Upon completion of hybridization, stringency washes were performed on the cells using the following procedure: three washes of fresh 50% formamide/2X SSC, pH 7.0, each for 5 minutes at 42°C, followed by one wash in 2X SSC, pH 7.0 for 5 minutes at 42°C, followed by one wash in 4X SSC/0.05% Tween 20, pH 7.0 for 5 minutes at 42°C and ending with one wash in 4X SSC/0.05% Tween 20, pH 7.0 for 5 minutes at room temperature. The cells were incubated in a solution of 4X SSC/0.05% Tween 20/0.05% blocker for 20 minutes at room temperature. While incubating, the conjugate mixes were prepared as follows:

Anti-DIG: 10µl anti-digoxygenin rhodamine, (200ug/ml) (Boehringer Mannheim)
490µl 4X SSC/0.05% Tween 20/0.05% blocker

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Avidin-FITC: 1µl fluorescent NeutrAvidin-FITC conjugate, (1mg/ml) (Molecular Probes)
499µl 4X SSC/0.05% Tween 20/0.05% blocker

Conjugate mixes were centrifuged for 5 minutes at 12,000 x g, and the supernatant was collected. Then, 10µl of each conjugate mix was added to the cells, the cells were covered with Parafilm M (American National Can) and incubated for 30 minutes in a dark, moist chamber at 37°C. Finally, the cells were washed in three washes of fresh 4X SSC/0.05% Tween 20, each for 5 minutes at room temperature. Once washing was complete, the slides were removed and excess fluid was drained. Ten microliters of diluted DAPI (4',6-diamidino-2-phenylindole) was applied to the slide, the cells were covered with a 22x22mm glass cover slip, and viewed using a Nikon fluorescent microscope with cooled CCD camera.

CALCULATION OF THE MITOTIC INDEX (MI)

% MI = (# cells in metaphase / total # of cells)*100

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ISOLATION OF RNA FROM BONE MARROW

The TRIZOL® procedure from Life Technologies was used in this study according to the manufacturer's directions. In brief, 0.5ml bone marrow sample was placed into a microcentrifuge tube. The cells were centrifuged and the supernatant was discarded. TRIZOL® Reagent was added followed by a brief incubation at room temperature. Then, chloroform (Fisher) was added and the sample was again incubated at room temperature, followed by centrifugation to separate the sample into distinct layers. The upper aqueous layer was recovered, and isopropyl alcohol (Fisher) was added to precipitate the RNA. The sample was briefly held at room temperature, followed by centrifugation. The supernatant was discarded and the RNA pellet was dried, and then re-dissolved in MBG water. The optical density, at 260nm, of the dissolved RNA was recorded using a spectrophotometer, and the RNA concentration was calculated from the relationship that 40µg/ml RNA = 1.00 OD_{260}.

REVERSE TRANSCRIPTION OF RNA

The commercially available reverse transcription system from Promega was used during this study according to the

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manufacturer's instructions. Briefly, MBG water, magnesium chloride, RT 10X Buffer, dNTP mixture, Recombinant RNasin® Ribonuclease Inhibitor, AMV Reverse Transcriptase, Oligo(dT)15 Primer, and 1.0µg of total RNA were combined. The sample was incubated at 42°C for 30 minutes, followed by a 5-minute incubation at 99°C, and another 5-minute incubation on ice. The newly synthesized cDNA was stored at -20°C until further use.

POLYMERASE CHAIN REACTION (PCR)

A set of two primers (Gibco BRL) were used to amplify the first 1214 base pairs of the *hBUB1* coding region. This area included the CD1 region and the NLS region. The primer set used was (Cahill, personal communication):

Primer 1 Forward 5'-CATGGACACCCCGGAAAATGTC-3'

Primer 2 Reverse 5'-GCATCTTTGCTGGCCACTGC-3'

Primer 1 was located at base pairs 49 through 70, and primer 2 was located at base pairs 1244 through 1263 in the consensus *hBUB1* sequence. PCR was accomplished using the Epicentre MasterAmp™ Taq DNA Polymerase and the following protocol, in brief, according to the manufacturer's instructions. MBG water, 10X PCR Buffer, magnesium chloride, dNTP, forward PCR primer, reverse PCR primer, Taq

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DNA Polymerase, and the cDNA template were combined in a PCR reaction tube. Seventy microliters of mineral oil was added to each PCR reaction tube, and the PCR reaction was completed using the following program specific for *hBUB1* PCR:

1. 95°C for 1 minute
2. 95°C for 30 seconds
3. 56°C for 30 seconds
4. 70°C for 2 minutes
5. Repeat to step 2, 35 times
6. 70°C for 5 minutes
7. Hold at 4°C

PCR results were viewed on a 1% agarose (Sigma) gel in 1X TBE (Tris-borate EDTA) stained with diluted ethidium bromide.

ISOLATION OF DNA FROM AN AGAROSE GEL

The Geneclean kit from BIO101 was used with the following protocol according to the manufacturer's directions. In brief, the PCR products were electrophoresed on a 1% agarose gel in 1X TAE (Tris-Acetate-EDTA), excised from the gel and weighed. 6M Sodium Iodide (NaI) was added, and the gel/NaI mixture was incubated in a 55°C water bath until

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the gel was completely dissolved. Glassmilk (BIO101) was added to each tube and the samples were vortexed, followed by a 5-minute incubation at room temperature and centrifugation. The supernatant was discarded and a series of three washes were performed. The washed glassmilk beads were air-dried, followed by the addition of TE buffer and incubation for 5 minutes at 55°C to elute the DNA. The glassmilk beads were pelleted by centrifugation, followed by recovery of the supernatant, which was stored at -20°C until further use.

LIGATION OF DNA INTO A PLASMID VECTOR

A commercial pGEM®-T vector ligation kit from Promega was used for this study with the following protocol using the manufacturer's instructions. Briefly, MBG water, 2X Rapid Ligation Buffer, pGEM®-T Vector, T4 DNA Ligase and PCR product DNA were combined and incubated for 1 hour at room temperature or overnight at 4°C. The ligated DNA was stored at -20°C until use.

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MEDIA PREPARATION

LB (Lennox) media was used throughout this study, both in broth and agar forms (preparation described in Chapter II, Solutions).

TRANSFORMATION OF LIGATED DNA

The procedure for transformation was as follows according to directions provided by Promega, with one exception noted below. LB/Amp/IPTG/X-Gal plates were prepared and brought to room temperature. The ligated PCR product DNA:pGEM®-T Vector was combined with High Efficiency JM109 Competent Cells followed by incubation on ice for 20 minutes. The cells were heat-shocked, followed by a 2-minute incubation on ice. LB broth was added to each tube, and the bacterial culture was incubated for 1 hour in a 37°C shaker. 0.5ml of the culture was spread onto a LB/Amp/IPTG/X-Gal plate (the amount of the culture that was spread onto the agar plate was changed from original protocol). The culture was incubated overnight in a 37°C incubator, and blue-white screening was performed to verify the presence of the inserted DNA within the bacterial vector.

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As an additional step to verify the presence of and the size of the inserted DNA, another round of PCR was performed using the bacterial DNA from one white colony as the template. PCR was performed using the protocol outlined in Chapter II, Polymerase Chain Reaction.

PLASMID DNA PURIFICATION

The QIAprep Spin Miniprep Kit from Qiagen was used to purify the plasmid DNA. The protocol was as per the manufacturer, and is outlined in brief. LB broth and Ampicillin (Sigma) were placed into a centrifuge tube, inoculated with one PCR-verified white bacterial colony, and incubated overnight in a 37°C shaker. The bacterial cells were pelleted, and lysed via alkaline lysis. The sample was centrifuged, and the supernatant was applied to a QIAprep spin column (a silica-based column included in the Qiagen kit). The column was centrifuged, followed by a series of washes to remove contaminants. Adding MBG water to the column and centrifuging eluted the DNA, which was stored at -20°C until use.

JM109 competent cells (Promega) were used in the transformation reaction. The JM109 cells have high levels of nuclease activity, which can interfere with subsequent

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sequencing reactions. However, one of the washing steps (above) removes excess nucleases, enabling the purified DNA to be used successfully in later sequencing reactions.

RESTRICTION ENZYME VERIFICATION

Prior to sequencing, a final verification was performed to ensure the proper size fragment was being sequenced. A restriction enzyme digest of the purified plasmid DNA was accomplished using SacI (Promega) and SacII (Promega) enzymes, which excised the insert from the vector without cutting the insert itself. The protocol was as follows according to the package insert provided by Promega. MBG water, plasmid DNA, 10X Multi-Core Buffer (Promega), Acetylated BSA (Promega), SacI and SacII were combined and the sample was incubated in a 37°C water bath for one hour. The restriction enzyme digest was viewed via electrophoresis on a 1% agarose gel in 1X TBE stained with ethidium bromide.

SEQUENCING OF THE *hBUB1* GENE

Sequencing was accomplished using an ALF Automated DNA Sequencer (Amersham Pharmacia Biotech). This sequencer

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reliably sequences 500 base pairs. Since the *hBUB1* RT-PCR product was 1.2kb, internal primers were incorporated to break the 1.2kb product into three 400 base pair products (Figure 7). These shorter products were easily sequenced by the ALF Automated DNA Sequencer. All primers were labeled with Cy-5 (sequencer requirement) and purified by the manufacturer (Integrated DNA Technologies, Inc.) using HPLC to remove unincorporated dideoxynucleotides. The primers used were as follows (Cahill, personal communication):

Primer 1 Forward 5′-AGGGTTTTCCCAGTCACGAC-3′

Primer 2 Forward 5′-CCTCTGTACATTGCCTGGGC-3′

Primer 3 Reverse 5′-TATTTCTGGGCTCTCAATTCTTC-3′

Primer 4 Reverse 5′-CACACAGGAAACAGCTATGAC-3′

Primers 1 and 4 were located within the plasmid vector sequence, primer 2 was located at base pairs 347 through 366 in the consensus *hBUB1* sequence and primer 3 was located at base pairs 803 through 825 in the consensus *hBUB1* sequence. Additionally, polyacrylamide gels designed for longer base pair sequencing were used (Repro-Gel Long Read by Amersham Pharmacia-Biotech). The Epicentre SequiTherm Excel II Long Read DNA Sequencing Kit for ALF DNA Sequencers was used for the sequencing reaction according to the manufacturer's directions with exceptions

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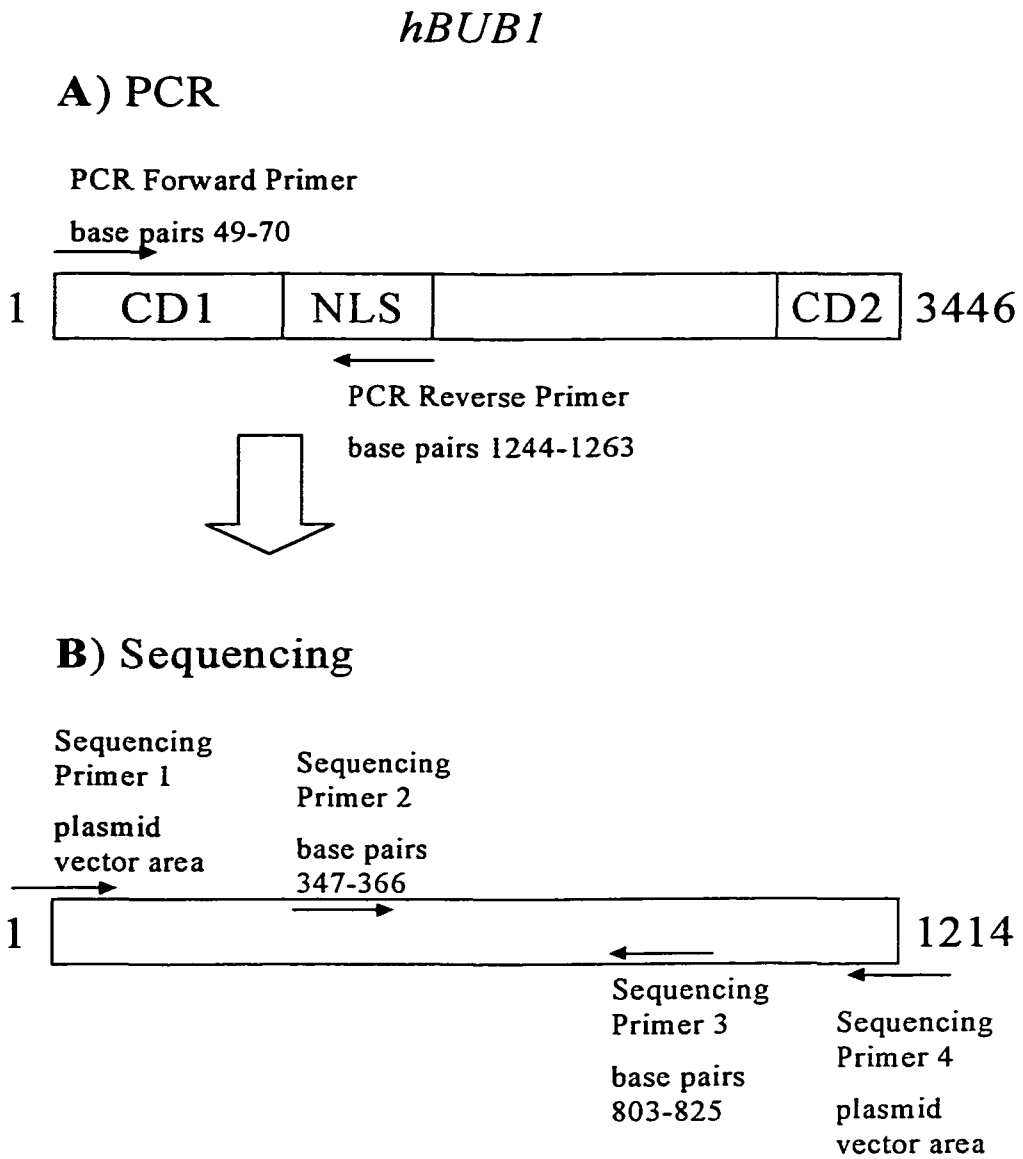

Figure 7. *hBUB1* PCR and Sequencing Primer Locations

(A) The PCR primers spanned the CD1 and NLS regions of the *hBUB1* gene. A total of 1214 base pairs were amplified by PCR.

(B) Sequencing was accomplished using a set of four primers.

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noted below. In brief, a stock DNA template solution of 100fmol/µl was prepared using the following two step formula: 1) the number of picomoles per microliter was calculated, using the following formula, where N = the number of base pairs in the insert:

$$[DNA]\mu g/\mu l \times 10^6 \times 1/660 \times 1/N = pmol/\mu l$$

2) one picomole of DNA was aliquoted into a microcentrifuge tube and MBG water was added to a final volume of 10µl, resulting in a stock solution of 100fmol/µl (the creation of a stock DNA template was not required in the original protocol). Then, MBG water, stock DNA template, sequencing buffer and labeled primer were combined to form a premix solution. For each reaction, four microcentrifuge tubes were labeled A,C,G or T. Termination Mixes were added to each of the four tubes, followed by the addition of the premix solution. Mineral oil was added to each tube, and the reactions were heated for 5 minutes at 95°C, followed by 30 PCR cycles according to the program provided my Epicentre. Stop/Loading Buffer was added to each reaction, and the reactions were incubated for 5 minutes at 95°C. Seven microliters of each reaction was loaded onto the sequencing gel (the amount loaded onto the sequencing gel was changed from the original protocol. The sequencer was run using the following parameters at constant power:

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1000 minute run

1500 volts

60 milliamps

25 watts

55°C

PCGene SEQUENCING ANALYSIS

The PCGene computer program was used to analyze all sequencing results. Alignment and translation of nucleotide sequences into corresponding amino acid sequences was also performed.

STATISTICAL ANALYSES

Statistical analyses were performed using the SPSS statistical analysis program. Two-sample t-tests and mulitivariate analysis of variances (ANOVA) were performed. Chi-squared analyses were computed using Microsoft Excel.

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CHAPTER III

RESULTS

SERIES A

Series A Patient Data

Series A consisted of patients with AA and controls. AA was chosen because of the controversy of whether or not AA is a pre-leukemic disorder. All bone marrow samples in series A underwent both FISH analysis for chromosomes 7 and 8 and sequencing of the *hBUB1* RT-PCR product. Bone marrow from 46 patients and six controls were analyzed in series A. Twenty-two out of 46 (47.8%) patients were male and the remainder (24; 52.2%) were female. The ethnic distribution of patients in series A was as follows: 4 patients (8.7%) were of Asian descent, 5 (10.9%) were African-American, 9 (19.6%) were Hispanic and 24 (52.2%) were Caucasian. In four AA patients, ethnicity was not reported (Figure 8).

All patients had a complete karyotype performed by the National Institutes of Health in Bethesda, Maryland (Table 1). All controls had a normal karyotype and no current or previous history of hematological disorders.

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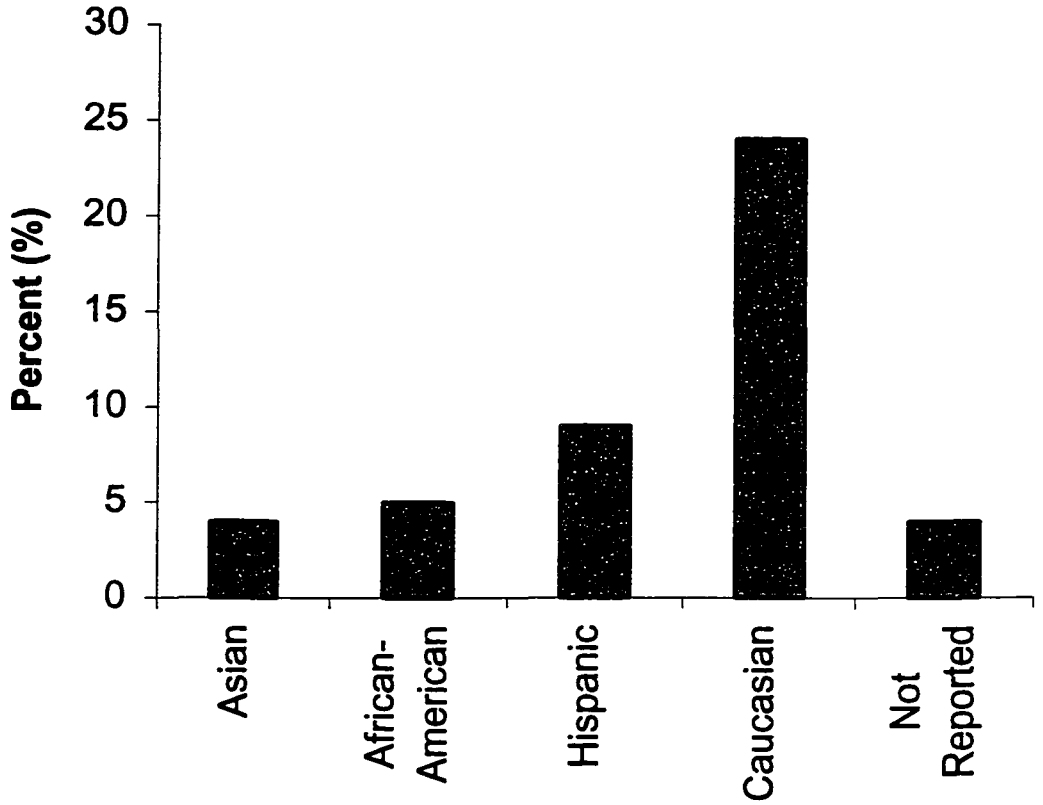

Figure 8. Ethnic Distribution of Patients in Series A

Series A consisted of patients with AA. Series A samples underwent both FISH for chromosomes 7 and 8 and sequencing of the *hBUB1* RT-PCR product.

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Table 1. Karyotype Analysis of Patient Samples in Series A

Patient #	Karyotype Result
29	46,xy
31	46,xy
33	46,xy
37	46,xy[29]
39	46,xy
40	46,xx[31]
41	46,xx
42	46,xy[30]
43	46,xx[30]
52	47,xy,+8[13]/46,xy[7]
57	46,xy
58	46,xy
60	46,x,ins(x;1)(p11.4;q21q42.3)[9]/46,xx[41]
64	46,xx[30]
77	46,xx[30]
81	46,xy
82	46,y,t(x;8)(p22.1;q24.1)
85	46,xx
86	46,xy
87	46,xx
88	46,xy[30]
90	46,xx
95	46,xx
98	46,xx[30]
99	47.xy,+8[14]/46,xy[7]
101	46,x,-y[14]/46,xy[6]
111	46,xy[30]
113	46,xx
115	46,xx
116	46,xx[30]
117	46,xx[30]
121	46,xy
128	46,xx[30]
129	46,xx[30]
130	46,xx
137	46,xx[30]
139	46,xx
142	46,xx[30]
146	46,xy[21]
147	46,xy[30]
153	46,xx
157	46,xy[30]
166	46,xy[30]

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Table 1 Continued.

Patient #	Karyotype Result
167	48,xxxc,+8[5]/47,xxxc[15]
168	Trisomy 8
169	46,xy

The patient number is listed with the corresponding karyotype analysis information. Seven patient samples (15.2%) showed an abnormal karyotype. The numbers in brackets [] indicate the number of cells counted.

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FISH Analysis for Chromosomes 7 and 8 on Series A Patient Samples and Control Samples

Nick translation was used to incorporate labeled nucleotides into DNA to be used as probes FISH. Fluorescently labeled alpha-satellite DNA probes were used to detect the centromeric region of chromosomes 7 and 8 in a two-probe, two-color FISH analysis. FISH was performed on all patient samples in series A; 300 nuclei were analyzed for each sample.

The nuclei were scored as "normal" if two signals for each chromosome were present. Occasionally, nuclei contained multiple small signals joined by a thin filamentous line (Figure 9). These occurrences were scored as one signal. In cells undergoing DNA replication (S phase), the centromeric region is elongated. Alpha-satellite DNA FISH probes bind to the centromeric region of the chromosomes, and therefore may exhibit the appearance of a thin filamentous line in cells currently undergoing S phase (Litmanovitch, et al., 1998; Mukherjee et al., 1992). Any signals less than 1 diameter apart from each other were scored as one signal; however, this was a rare occurrence. To allow unbiased scoring, an independent person coded all samples. The code was broken only after the entire set of samples was scored. Each set included at least one control

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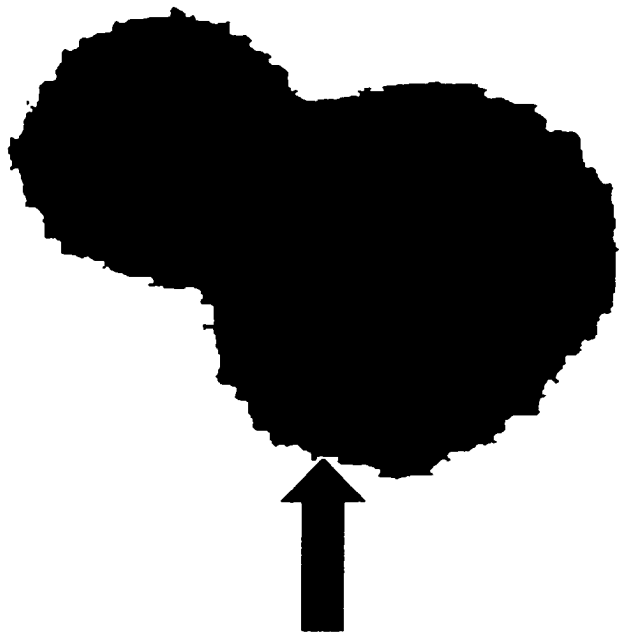

Figure 9. FISH Analysis for Chromosome 7

Example of a normal cell containing two copies of chromosome 7, with one copy presented as multiple small signals joined by a thin line (arrow). In this example, both cells were scored as normal, with each cell containing two copies of chromosome 7.

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and one patient sample.

Two out of 46 (4.3%) patient samples showed statistically significantly elevated levels of monosomy 7 (Figure 10, Table 2) when compared to the control sample value of 1.55% monosomy 7. Bone marrow from patient 111 showed 4.0% monosomy 7, and bone marrow from patient 128 showed 5.17% monosomy 7. There were no patient samples exhibiting statistically significant levels of trisomy 7, where the control sample value was 0.46% trisomy 7. Two out of 46(4.3%) patient samples showed statistically significantly elevated levels of monosomy 8 (Table 2) when compared to the control sample value of 2.13% monosomy 8. Bone marrow cells from patient 58 had 5.3% monosomy 8, while bone marrow cells from patient 128 had 12.1% monosomy 8. Four out of 46 (8.7%) patient samples showed statistically significantly elevated levels of trisomy 8 (Table 2) when compared to the control sample value of 0.98% trisomy 8. Bone marrow samples from patient 52 had 20.3% trisomy 8, patient 99 had 29.3% trisomy 8, patient 116 had 3.3% trisomy 8 and patient 168 had 10.3% trisomy 8. Bone marrow cells from patient 128 showed a high level of abnormality in both chromosomes 7 and 8 (monosomy 7 and monosomy 8).

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Figure 10. FISH Analysis Showing Apparent Monosomy 7

This photograph shows the detection of monosomy 7 using FISH in a metaphase cell. Only one signal is shown for chromosome 7 (arrow), indicating apparent monosomy for that chromosome.

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Table 2. Patient Samples in Series A Showing Elevated Levels of Cytogenetic Abnormalities with Control Data

Patient #	% Monosomy 7	% Trisomy 7	% Monosomy 8	% Trisomy 8
52	2.67	0.67	0.33	20.33*
58	2.00	0.00	5.33*	0.00
99	0.67	0.00	2.67	29.33*
111	4.00*	0.67	4.33	2.67
116	2.67	1.00	1.00	3.33*
128	5.17*	0.00	12.07*	1.72
168	1.67	0.00	4.00	10.33*

*Statistically Significant

Control #	% Monosomy 7	% Trisomy 7	% Monosomy 8	% Trisomy 8
Con 1	0.66	1.33	1.66	0.66
Con 2	2.29	0.76	6.10	1.52
Con 5	2.00	0.66	1.33	0.66
Ded 4	1.66	0.00	1.00	1.33
Ded 5	1.00	0.00	0.33	1.00
Ded 6	1.66	0.00	2.33	0.66

Percent aneuploidy for chromosomes 7 and 8 for patient samples in series A, versus control sample data, for patient samples that showed a statistically significant level of aneuploidy.

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In comparison with data obtained by karyotype analysis, FISH detected abnormalities not detected by karyotype in 4 of 7 patient samples (Table 3). This lead to the conclusion that FISH is approximately twice as sensitive in detecting cytogenetic abnormalities than karyotype analysis in this study.

Analysis of the Mitotic Index (MI) in Patient Samples from Series A and Control Samples

The MI is an indication of the cell cycle frequency. For this study, the MI was determined by counting the number of cells in metaphase and dividing by the total number of cells scored. Cells were determined to be in metaphase if individual chromosomes were visible (such as Figure 10). The MI was examined since, hypothetically, *hBUB1* would have a higher level of expression in a population of cells undergoing rapid division. For this study, the MI was calculated (Chapter II) simultaneously while performing FISH analysis on unstimulated bone marrow cells. The average MI for control samples was 0.33%, while the average MI for patient samples in series A was 0.44%. There was no statistically significant difference between the patient and control samples ($p=0.571$). A rapidly dividing cell line, HL60, assayed by Dr. Michael Stacey, was found to

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Table 3. Karyotype versus FISH Results in Series A Patients Showing Elevated Levels of Aneuploidy

Patient #	FISH Result	Karyotype Result
52	+8 (20.33%)	47,xy,+8[13]/46,xy[7]
58	-8 (5.33%)	46,xy
99	+8 (29.33%)	47.xy,+8[14]/46,xy[7]
111	-7 (4.00%)	46,xy[30]
116	+8 (3.33%)	46,xx[30]
128	-7 (5.17%)	46,xx[30]
	-8 (12.07%)	
168	+8 (10.33%)	trisomy 8 (exact number not available)

Samples from patients 58, 111, 116 and 128 showed elevated levels of chromosome abnormalities for chromosomes 7 and/or 8. In these patients, the abnormalities were not detected by karyotype analysis. Numbers in brackets [] indicate the number of cells.

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have a MI of 2.2% in exponentially growing cells, six times higher than the control sample MI rate in this study (Stacey, personal communication). For this reason, HL60 cDNA was used as the positive control for the *hBUB1*-specific RT-PCR. It is known that hBUB1 is more abundant during active cell division, therefore, a cell line (such as HL60) with an increased number of cell divisions would have elevated expression of *hBUB1*, allowing for easy amplification in a RT-PCR reaction.

hBUB1-Specific RT-PCR on RNA from Patient and Control Samples in Series A

PCR was performed on cDNA, constructed from total RNA, for all patients in series A. Ten out of 46 (21.7%) series A patient cDNA samples amplified the expected 1.2kb *hBUB1* RT-PCR product (Figure 11). cDNA from each patient sample underwent two separate, but identical rounds of *hBUB1*-specific PCR for verification. All 36 patient cDNA samples showing no *hBUB1* RT-PCR product were re-analyzed by an additional round of PCR using primers for the ubiquitously expressed gene actin. All 36 patient cDNA samples (100%) amplified the expected ~550 base pair actin PCR product, verifying that each RNA sample was intact and not degraded (Figure 12).

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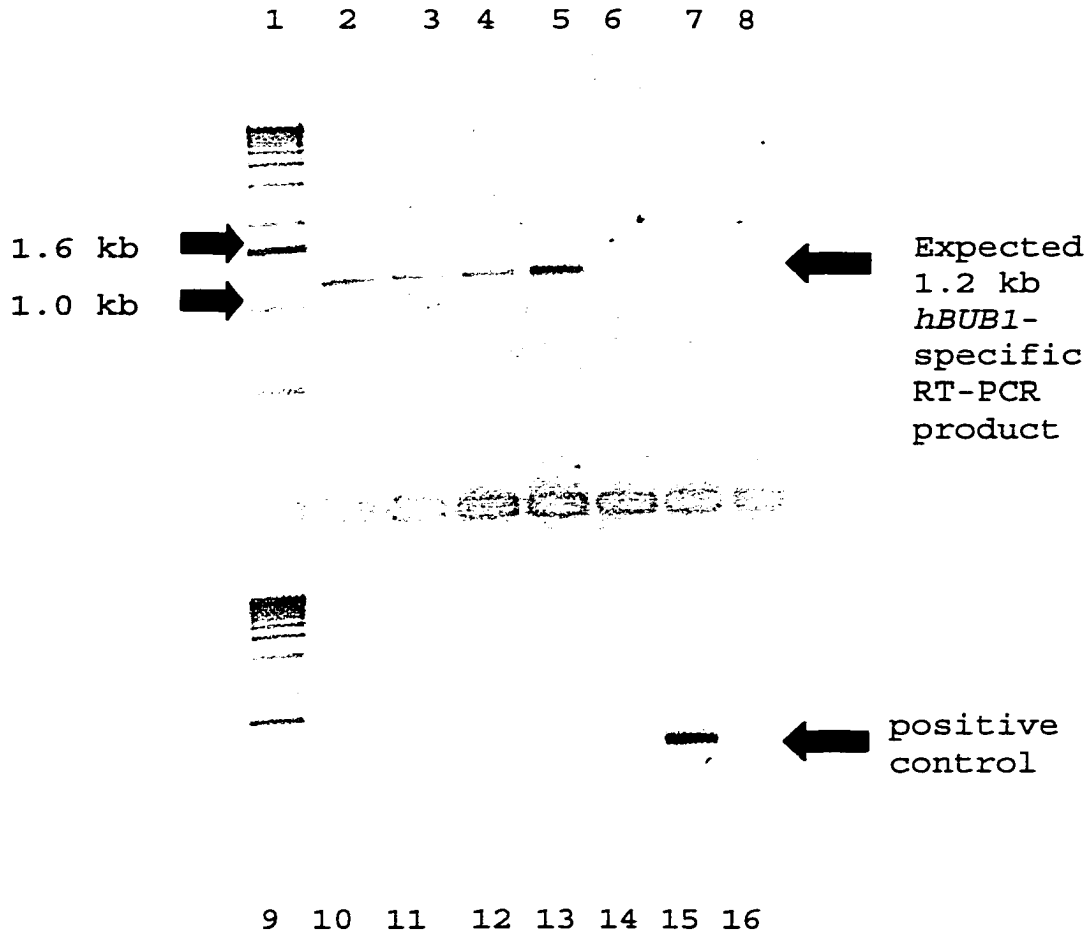

Figure 11. Gel Electrophoresis of *hBUB1*-specific RT-PCR.

Lanes 1 and 9 contain a 1 kb DNA ladder. Lanes 2-5 contain patient cDNA samples showing the expected *hBUB1*-specific RT-PCR product of 1.2kb. Lanes 10-14 contain patient cDNA samples showing no amplification of the *hBUB1*-specific RT-PCR product. Lane 15 contains the *hBUB1*-specific RT-PCR reaction completed on cDNA from HL60, which was used as the positive control. Lane 16 contains the negative control, and lanes 6-8 are empty.

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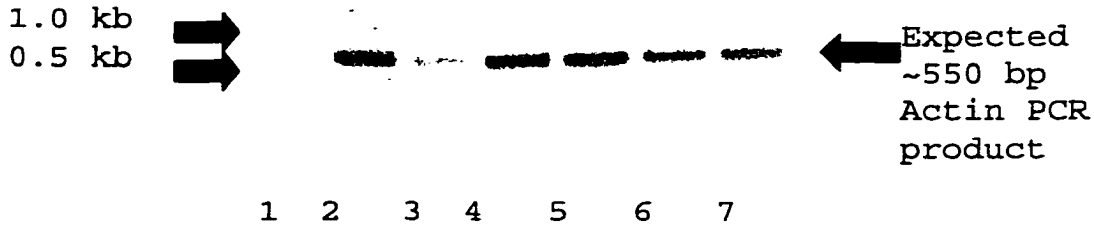

Figure 12. Gel Electrophoresis of Actin PCR.

Lane 1 contains a 1 kb DNA ladder. Lanes 2-7 contain patient cDNA samples showing the expected Actin PCR product of ~550 base pairs.

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All 36 patient RNA samples that showed no *hBUB1* RT-PCR product underwent a second round of cDNA synthesis. In the second cDNA synthesis, an *hBUB1* oligonucleotide reverse PCR primer (spanning base pairs 1195-1215 in the normal consensus sequence) was used in place of the Oligo$(dT)_{15}$ primer. This was performed to ensure that the cDNA synthesis began at the 3′ end of the NLS region within the *hBUB1* RNA, thereby increasing the likelihood of successful cDNA synthesis provided that *hBUB1* was being expressed. This ensured that cDNA synthesis could occur even if the *hBUB1* RNA past the 3′ end of the NLS region was degraded. The cDNA synthesized using the *hBUB1* oligonucleotide reverse PCR primer was subjected to a new round of *hBUB1*-specific PCR. Once again, all 36 patient cDNA samples showed no amplification of the *hBUB1* RT-PCR product (Figure 13). HL60, the rapidly dividing cell line, chosen as the PCR positive control, consistently amplified the correct 1.2kb *hBUB1* RT-PCR product (Figure 11).

cDNA was synthesized from total RNA isolated from five control bone marrow samples using the Oligo$(dT)_{15}$ primer. *hBUB1*-specific PCR was performed on the cDNA, and three out of 5 samples (60%) amplified the correct 1.2kb *hBUB1* RT-PCR product. The remaining two showed no *hBUB1* RT-PCR product. Due to lack of material, the control samples did

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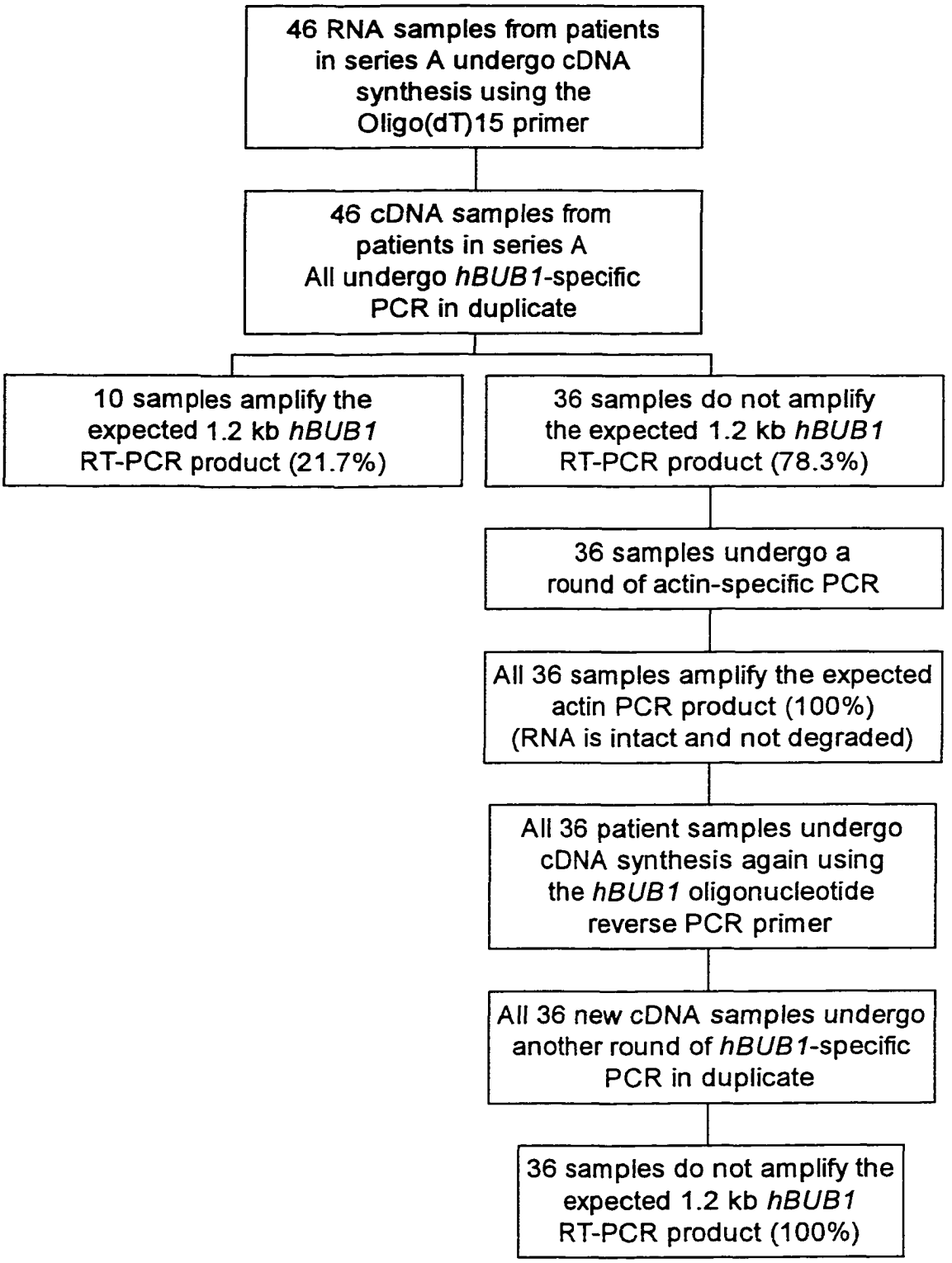

Figure 13. Flow Chart for cDNA Synthesis and *hBUB1*-specific RT-PCR in Patient Samples from Series A

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not undergo PCR for actin or additional cDNA synthesis using the *hBUB1* oligonucleotide reverse PCR primer. To provide more accurate results, the number of control samples should be increased, however only five control samples were available at the time of this study.

Sequencing of the *hBUB1* RT-PCR Product in Patient Samples from Series A and Sequencing of the HL60 RT-PCR Positive Control

The cDNA from the ten patient samples in series A that exhibited the correct 1.2kb *hBUB1* RT-PCR product were sequenced to look for base pair changes within the *hBUB1* CD1 and NLS regions. In addition, cDNA from the PCR positive control HL60 was also sequenced. Sequencing was performed first 1214 base pairs, which spanned the first conserved domain and the nuclear localization sequence in the *hBUB1* gene (Figure 14). *hBUB1* is comprised of 3446 base pairs, and consists of 25 exons. All base pair changes are referenced according to their base pair location within the entire *hBUB1* consensus sequence, as reported by Cahill et al. (1998).

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CATGGACACCCCGGAAAATGTCCTTCAGATGCTGGTGAATGGGAAAGATACATACAGTG
GGTAGAAGAGAATTTTCCTGAGAATAAAGAATACTTGATAACTTTACTAGAACATTTAA
TGAAGGAATTTTTAGATAAGAAGAAATACCACAATGACCCAAGATTCATCAGTTATTGT
TTAAAATTTGCTGAGTACAACAGTGACCTCCATCAATTTTTTGAGTTTCTGTACAACCA
TGGGATTGGAACCCTGTCATCCC**CTCTGTACATTGCCTGGGC**GGGGCATCTGGAAGCCC
AAGGAGAGCTGCAGCATGCCAGTGCTGTCCTTCAGAGAGGAATTCAAAACCAGGCTGAA
CCCAGAGAGTTCCTGCAACAACAATACAGGTTATTTCAGACACGCCTCACTGAAACCCA
TTTGCCAGCTCAAGCTAGAACCTCAGAACCTCTGCATAATGTTCAGGTTTTAAATCAAA
TGATAACATCAAAATCAAATCCAGGAAATAACATGGCCTGCATTTCTAAGAATCAGGGT
TCAGAGCTTTCTGGAGTGATATCTTCAGCTTGTGATAAAGAGTCAAATATGGAACGAAG
AGTGATCACGATTTCTAAATCAGAATATTCTGTGCACTCATCTTTGGCATCCAAAGTTG
ATGTTGAGCAGGTTGTTATGTATTGCAAGGAGAAGCTTATTCGTGGGGAATCAGAATTT
TCCTTT**GAAGAATTGAGAGCCCAGAAATA**CAATCAACGGAGAAAGCATGAGCAATGGGT
AAATGAAGACAGACATTATATGAAAAGGAAAGAAGCAAATGCTTTTGAAGAACAGCTAT
TAAAACAGAAAATGGATGAACTTCATAAGAAGTTGCATCAGGTGGTGGAGACATCCCAT
GAGGATCTGCCCGCTTCCCAGGAAAGGTCCGAGGTTAATCCAGCACGTATGGGGCCAAG
TGTAGGCTCCCAGCAGGAACTGAGAGCGCCATGTCTTCCAGTAACCTATCAGCAGACAC
CAGTGAACATGGAAAAGAACCCAAGAGAGGCACCTCCTGTTGTTCCTCCTTTGGCAAAT
GCTATTTCTGCAGCTTTGGTGTCCCCAGCCACCAGCCAGAGCATTGCTCCTCCTGTTCC
TTTGAAAGCCCAGACAGTAACAGACTCCATGTTTGCAGTGGCCAGCAAAGATGC

Figure 14. Consensus Sequence of the *hBUB1* RT-PCR Product

Underline indicates a PCR primer location. Bold indicates an internal sequencing primer location.

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Initially, direct-from-PCR sequencing was attempted to reduce artifacts and preparation time. However, results from direct-from-PCR sequencing reactions were of poor quality when compared to results obtained by cloning first. For that reason, cloning prior to sequencing was used for this study. Sequencing was accomplished by cloning the PCR product into a plasmid vector, transformation of the ligated PCR product and purification of the plasmid DNA. However, a disadvantage encountered when cloning each colony contains only one of the two alleles. Therefore, each sequence obtained represents only one of the two *hBUB1* alleles present. This issue can be resolved by sequencing multiple colonies from the same reaction to ensure that both alleles are sequenced.

Due to variations in the amount of extracted nucleic acids, each sample underwent a different number of reverse transcription reactions, PCR reactions and/or sequences. Ideally, each individual patient RNA sample would have undergone five separate reverse transcription reactions. Each of the five resulting cDNAs (per patient) would have undergone separate rounds of *hBUB1*-specific PCR. Finally, each PCR reaction would have been separately cloned and sequenced. The end result would have been five separate sequences for each patient sample, and a confidence

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interval of 80%. Statistically, 20 separate cDNA syntheses, PCR reactions and sequences should be performed on each patient sample to establish a confidence interval of 95% (Cahill, personal communication). Artifacts can be introduced at the reverse transcription stage, the PCR stage, or during sequencing. However, by increasing the number of RT-PCR and sequence analyses, the likelihood of error is reduced. The maximum possible number of RT-PCR and sequencing replications were performed on each patient sample in an effort to rule out potential artifacts. Each patient sample underwent between one and seven RT-PCR and sequencing reactions, depending on the sample availability. In addition, since each bacterial colony contains just one of the two *hBUB1* alleles, every time a colony was sequenced, there was a 50/50 possibility of sequencing either allele. In the instances where only one sequence determination was performed and shown to be normal, it is possible that the other allele could have been mutant, and vice versa. In these cases, the results obtained were inconclusive.

RNA from patient 42 underwent one round of RT-PCR and sequencing. Since only one RT-PCR and sequencing reaction was performed, the power of the results was reduced, and therefore the results should be evaluated cautiously.

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Sequencing of *hBUB1* in the RT-PCR product sample from patient 42 indicated two base pair changes located at base pairs 824, 924 and an insertion. The insertion consisted of 11 extra base pairs inserted within the oligonucleotide forward PCR primer sequence. The significance of this insertion, if any, is unknown. Due to the lack of additional sequence information, the preliminary results for patient 42's sample are inconclusive (Figure 15, Table 4).

RNA from patient 43 underwent one round of RT-PCR and sequencing. Sequencing of the *hBUB1* RT- product indicated two base pair changes at base pairs 845 and 1170. Again, due to the lack of additional sequence data, concrete conclusions cannot be drawn (Figure 16, Table 4).

RNA from patient 52 underwent two separate rounds of RT-PCR (PCR #1 and #2). When sequenced, the DNA recovered from PCR #1 showed no base pair changes, while the DNA recovered from PCR #2 showed a base pair change at base pair 472 and an insertion/deletion. The insertion was very similar to the insertion found in patient sample 42, and consisted of 12 extra base pairs inserted within the oligonucleotide forward PCR primer sequence, followed by a five base pair deletion. The significance of this insertion/deletion, if any, is unknown. A preliminary

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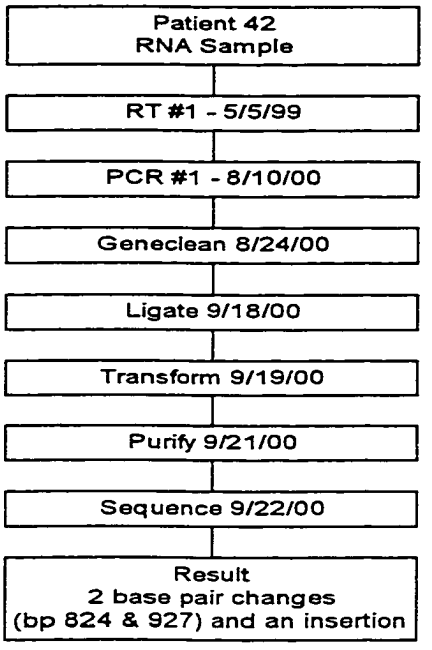

Figure 15. Sequencing Flow Chart for Patient 42

Preliminary results indicate two base pair changes and an insertion. However, these sequencing results were not duplicated.

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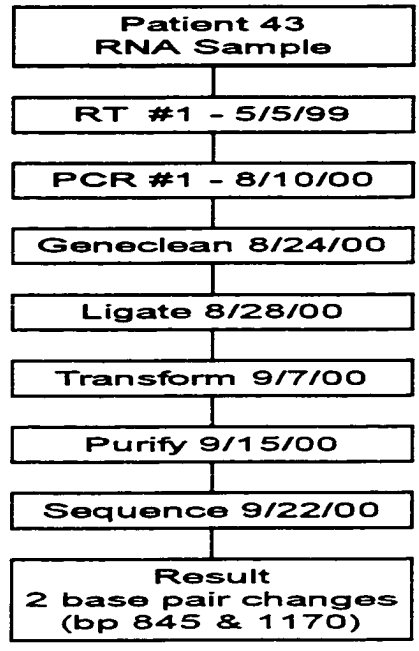

Figure 16. Sequencing Flow Chart for Patient 43

Preliminary results indicate two base pair changes. However, these sequencing results were not duplicated.

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observation is that the base pairs inserted within the forward primer area in both patient samples are very similar to the base pairs from the plasmid vector, which can be found immediately prior to the forward primer starting point in the normal consensus sequence. Perhaps there was an error during ligation in which the ligation area on the 5′ end of the RT-PCR product was disturbed, or the insertion(s)/deletion may be due to a polymerase error. Due to the lack of additional sequence information, the preliminary results for patient 52's sample are inconclusive (Figure 17, Table 4).

The RNA from patient 82 underwent two rounds of reverse transcription. The product from the first reverse transcription reaction underwent one round of PCR (PCR #1), followed by cloning and sequencing. The product from the second reverse transcription reaction underwent six separate PCR reactions (PCR #2-#7), followed by cloning and sequencing. The DNA recovered from PCR #1 was sequenced five times, which ruled out the possibility of a sequencing artifact, however, the DNA from PCR #2-#7 was only sequenced once. The sequencing results from PCR #1 showed four base pair changes located at base pairs 418, 532, 632 and 908. The sequencing results from PCR #3 showed two base pair changes located at base pair 711 and 1107. The

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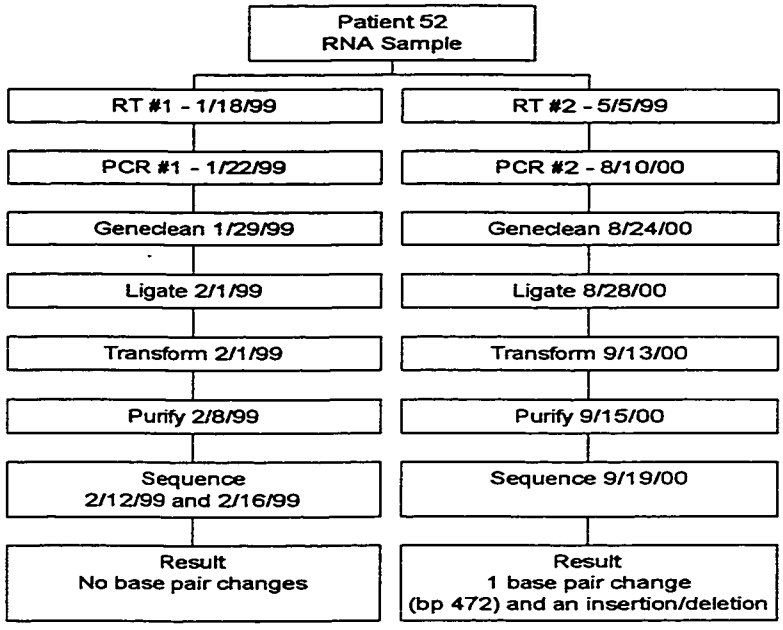

Figure 17. Sequencing Flow Chart for Patient 52

Preliminary results indicate one base pair change and an insertion/deletion in the sequencing of DNA from PCR #2, while the other sequence was normal, making the results inconclusive.

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sequencing results from PCR #4 showed one base pair change at base pair 1177. The remaining four PCR reactions showed no base pair changes within their sequences. None of the base pair changes were seen in duplicate, and were most likely artifacts. The RNA from patient 82 underwent the most sequencing reactions (seven in total) out of the ten patient samples analyzed from series A. The high level of sequence variation in this patient sample showed that the potential for artifacts was high among these experiments. The overall sequencing data from patient 82's sample indicated that the majority of sequences performed had no base pair changes detected. Therefore, it is reasonable to conclude that the remaining sequence variations were artifacts (Figure 18, Table 4).

RNA from patient 90 underwent two rounds of RT-PCR and sequencing. Sequencing of both RT-PCR products showed no base pair changes within the *hBUB1* sequence. Therefore, it is reasonable to conclude, preliminarily, that the sequence data obtained from patient 90 is identical to the normal consensus sequence data reported by Cahill et al (1998) (Figure 19, Table 4).

RNA from patient 117 underwent one round of reverse transcription followed by two separate PCR analyses. Both PCR products were cloned and sequenced. The product from

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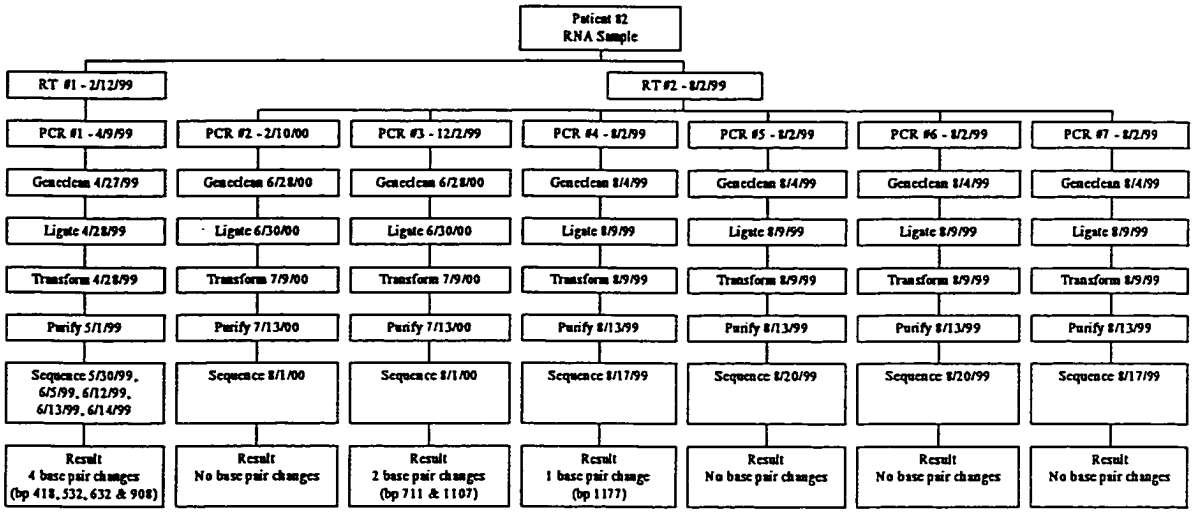

Figure 18. Sequencing Flow Chart for Patient 82

Preliminary results indicate four base pair changes in the sequence from PCR #1, 2 base pair changes in the sequence from PCR #3, and 1 base pair change in the sequence from PCR #4, all of which are different. The remaining sequencing reactions showed no base pair changes. These results are contradictory.

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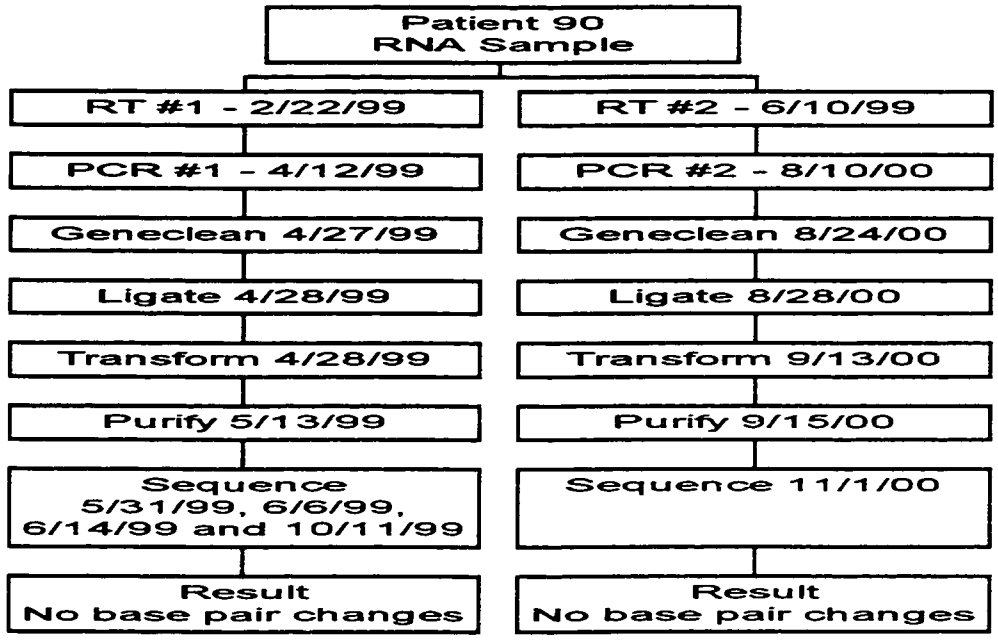

Figure 19. Sequencing Flow Chart for Patient 90

Preliminary results showed both sequences with no base pair changes, indicating that the sequence was the same as the normal consensus sequence reported by Cahill et al., 1998.

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PCR #1 was sequenced six times and preliminary results indicated that there were no base pair changes present in any of the six sequencing reactions. All six sequencing reactions were performed on DNA from a single colony. The second PCR product was sequenced once, and one base pair change was observed at base pair number 1055 in the *hBUB1* normal consensus sequence. The sequencing results were conflicting, showing a 50% probability that the sequence obtained was normal and a 50% probability that the sequence obtained contained a base pair change. Considering the high risk of artifacts in this study, it is possible that the base pair change seen is in fact an error. However, at this point, the data is inconclusive (Figure 20, Table 4).

RNA from patient 121 underwent three separate rounds of RT-PCR (PCR #1-#3). The DNA recovered from PCR #1 and #3 showed no base pair changes upon sequencing; however, the DNA recovered from PCR #2 showed two base pair changes at base pairs 615 and 1151. The majority of the sequencing data indicated that no base pair changes were present, therefore there is a high probability that the two base pair changes observed are artifacts. It is reasonable to conclude that patient 121's sample is most likely normal, with no base pair changes present (Figure 21, Table 4).

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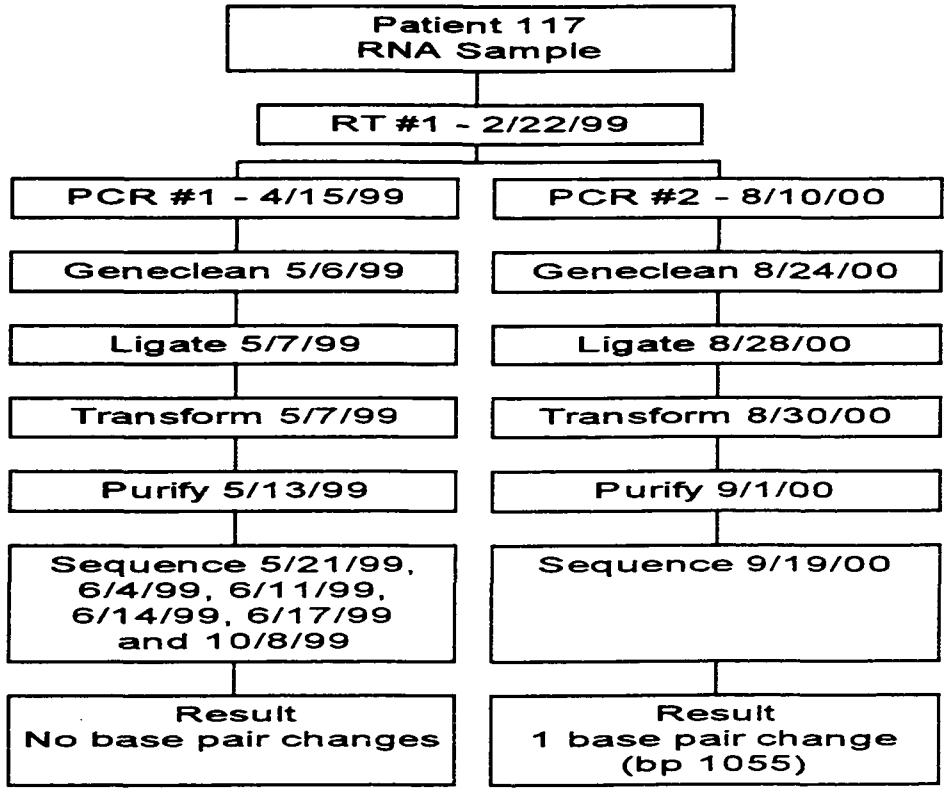

Figure 20. Sequencing Flow Chart for Patient 117

Preliminary results indicate one base pair change in the sequence from PCR #2, with the other sequence as normal, making the results inconclusive.

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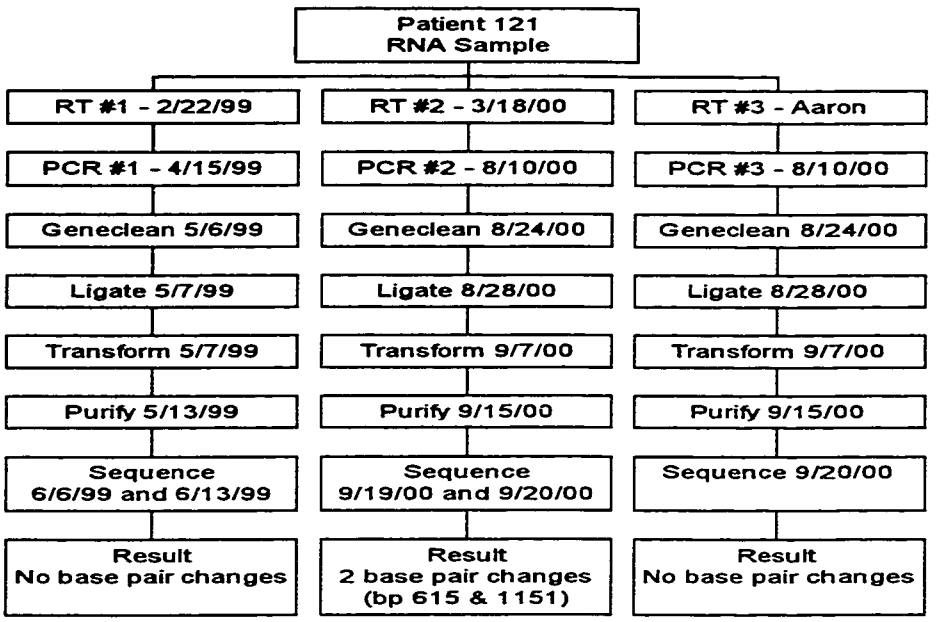

Figure 21. Sequencing Flow Chart for Patient 121

Preliminary results indicate two base pair changes in the sequence from PCR #2, and no base pair changes in the other two sequences, leading to the conclusion that the sequence is most likely normal.

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The RNA from patient 129 underwent one round of RT-PCR and sequencing. Sequencing results showed no base pair changes were present. As with all patient samples that were sequenced only once, the results are inconclusive (Figure 22, Table 4).

RNA from patient 137 underwent two rounds of reverse transcription. The product from the first reverse transcription reaction subsequently underwent two separate rounds of PCR (PCR #1 and #2), while the product from the second reverse transcription reaction underwent only one round of PCR (PCR #3). When sequenced, the DNA recovered from PCR #1 showed no base pair changes present. DNA from PCR #2 showed five base pair changes located at base pairs 327, 844, 868, 975 and 1227. The sequencing results from the DNA recovered from PCR #3 showed one base pair change at base pair 470. As with patient 82, none of the base pair changes were present in duplicate. In addition, the probability of six base pair changes occurring within 1214 base pairs is rare, and would indicate an extremely high mutation rate. If that were the case, the base pair changes would have been detected in other samples and found in duplicate. The actual probability of this many different base pair changes occurring is very low; therefore, the base pair changes seen are most likely

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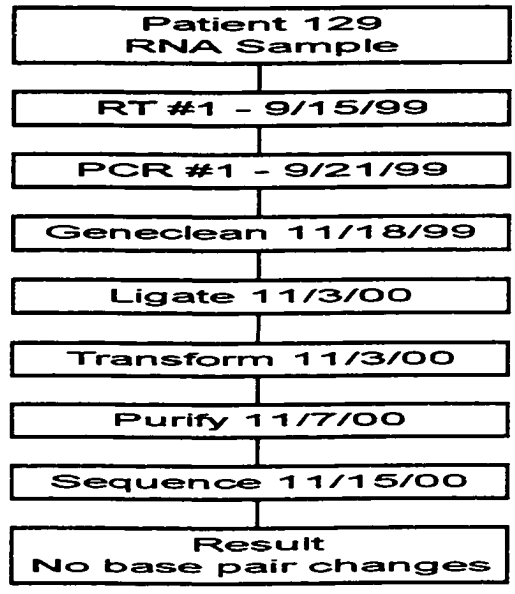

Figure 22. Sequencing Flow Chart for Patient 129

Preliminary results indicate no base pair changes; however, the lack of additional sequence information makes the results inconclusive.

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artifacts. Nevertheless, the sequencing data for patient 137 is conflicting, and a concrete conclusion cannot be *MADe* (Figure 23, Table 4).

The RNA from patient 153 underwent one round of RT-PCR and sequencing. The sequence data for patient 153 showed five base pair changes located at base pairs 645, 930, 931, 932 and 933. The sequential base pair changes were unique to patient 153, and were not seen elsewhere. Due to the lack of additional sequencing data, these results are inconclusive (Figure 24, Table 4).

Sequencing of the HL60 *hBUB1*-specific RT-PCR product revealed no base pair changes, and was consistent with the normal consensus sequence reported by Cahill et al. (1998). Any potential polymorphism or mutation that does not result in a change in the amino acid sequence, does not affect the protein sequence. Therefore, any base pair change that does not have a corresponding amino acid change would most likely be inconsequential.

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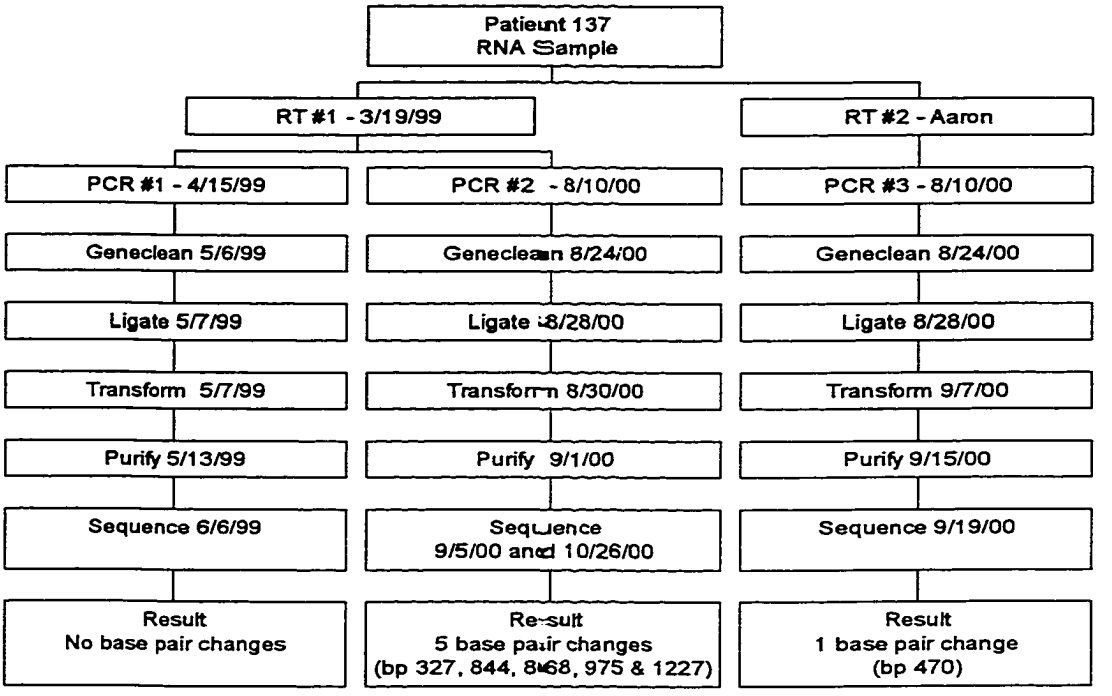

Figure 23. Sequencing Flow Chart for Patient 137

Preliminary results indicate five base pair changes in the sequence from PCR #2, 1 base pair change in the sequence from PCR #3 (all different), and no base pair changes in the sequence from PCR #1, making the results conflicting and inconclusive.

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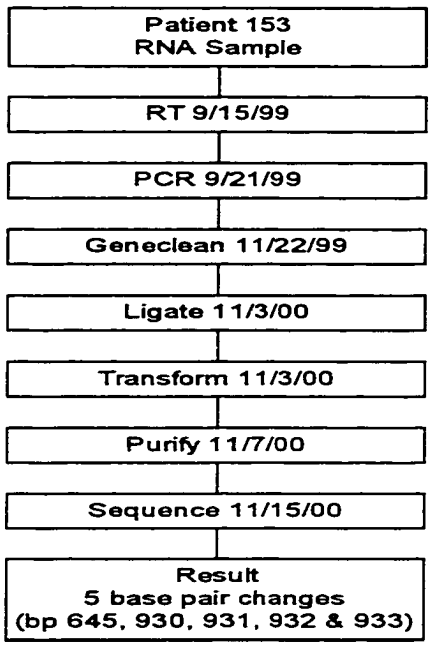

Figure 24. Sequencing Flow Chart for Patient 153

Preliminary results indicate five base pair changes; however, the lack of additional sequence information makes the results inconclusive.

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Table 4. Summary of Base Pair Changes/Amino Acid Changes in Patient Samples Sequenced from Series A with Corresponding FISH Analysis

Patient #	FISH Result	PCR#	BP#	BP Change	Amino Acid Change
42	Normal	#1	ins	11 bp ins	Protein unable
			824	T→C	to be
			937	T→C	translated
43	Normal	#1	845	G→A	Glu→Lys
			1170	T→C	No change
52	+8 (20.33%)	#2	ins/ del 472	12 bp ins/5 bp del G→A	Protein unable to be translated
82	Normal	#1	418	G→A	No change
			532	T→C	No change
			632	G→A	Gly→Arg
			908	C→T	Gln→stop
		#3	711	T→A	Val→Glu
			1107	A→G	No change
		#4	1177	A→C	No change
117	Normal	#2	1055	A→T	Arg→stop
121	Normal	#2	615	A→G	Gln→Arg
			1151	G→A	No change
139	Normal	#2	327	G→A	Gly→Glu
			844	T→C	No change
			868	A→G	No change
			975	A→G	Glu→Gly
			1227	T→C	Val→Ala
		#3	470	A→G	Arg→Gly
153	Normal	#1	645	C→T	Ser→Leu
			930	A→G	\
			931	T→C	MetAspGluLeu→
			932	G→C	MetGlyArgLeu
			933	A→G	/

Comparison of FISH data and sequencing results for *hBUB1*. Abbreviations: BP=base pair; ins=insertion; del=deletion

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Statistical Analyses on Data from Patient Samples in Series A

Statistical analyses were performed using the SPSS computer software program. The data obtained from the FISH analyses for aneuploidy rates in chromosomes 7 and 8 were evaluated using a two-sample t-test. The results showed that there was no statistically significant difference between the average level of aneuploidy in the patient samples versus the control samples ($p=0.453$ for monosomy 7, $p=0.194$ for trisomy 7, $p=0.840$ for monosomy 8 and $p=0.685$ for trisomy 8).

To allow comparison of individual patient data and control data, a chi-squared analysis was performed. The results from the chi-squared analysis show that two out of 46 patients (4.3%) showed statistically significantly higher levels of monosomy 7, two out of 46 patients (4.3%) showed statistically significantly higher levels of monosomy 8 and four out of 46 patients (8.7%) showed statistically significantly higher levels of trisomy 8. One patient sample showed elevated levels of both monosomy 7 and monosomy 8 (2.2%) (outlined previously in Table 2).

A statistical comparison was performed to check the effect of age, race, and responsiveness to treatment with respect to aneuploidy. An analysis of variance was

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performed using the SPSS computer program, and the only statistically significant result showed that African-American males were more likely to exhibit monosomy for chromosome 8 ($p=0.016$), however the sample size was low. Responsiveness to treatment and age of the patient did not correlate with the level of aneuploidy for either chromosome 7 or 8.

SERIES B

Series B Patient Data

One hundred and forty-three additional patient samples underwent RT-PCR and sequencing analysis for *hBUB1* without FISH analysis. The patient samples in series B consisted of 49 patients with AA, 63 patients with MDS, 8 patients with pancytopenia, 6 patients with PNH, 3 patients with anemia, 3 patients with PRCA, 3 patients with thrombocytopenia, 2 patients with myeloproliferative disease, 2 patients with neutropenia, 1 patient with porvovirus, 1 patient with LGL, 1 patient in transformation to leukemia and 1 patient with "liver transplant" as the diagnosis (Figure 25). The sex of the patient was known for 136 out of 143 patients; 52 out of 143 (36.4%) were female and 85 out of 143 (59.4%) were male. The ethnic

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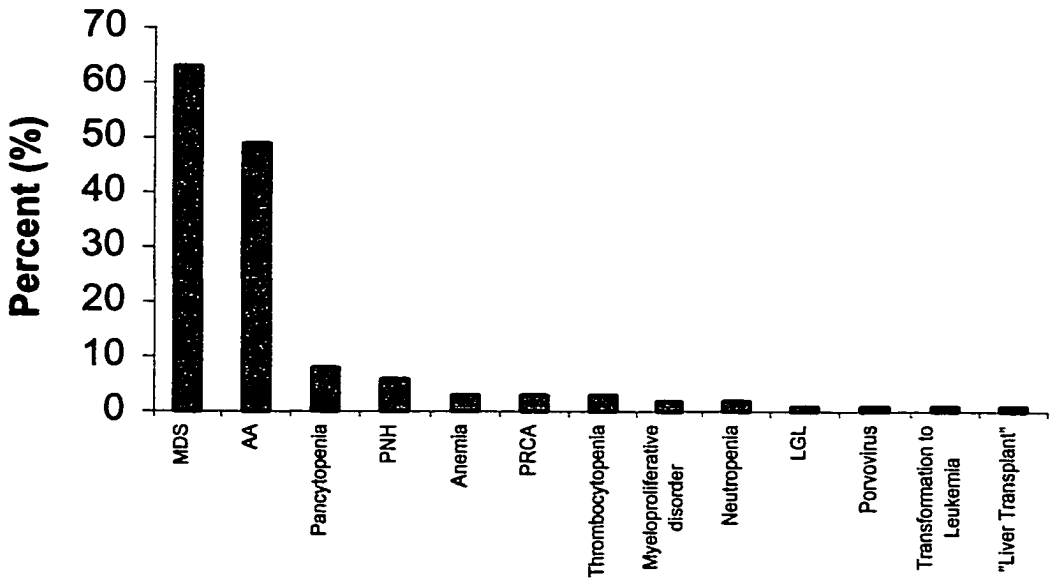

Figure 25. Distribution of Bone Marrow Disorders in Series B Patients

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distribution of patients in series B showed that eight out of 143 (5.6%) were Asian, ten out of 143 (7.0%) were African-American, 13 out of 189 (9.1%) were Hispanic and 82 out of 143 (57.3%) were Caucasian. In 30 patients (21.0%), ethnicity was not reported (Figure 26). Sixty-eight patients out of 143 (47.6%) had a complete karyotype performed by the National Institutes of Health in Bethesda, Maryland (Appendix A). The remaining 75 patients did not undergo karyotypic analysis.

***hBUB1*-Specific PCR on cDNA from Patients in Series B**

PCR was performed on cDNA constructs from all 143 patient samples. Twenty-one out of 143 (14.7%) patient samples amplified the expected 1.2kb *hBUB1* RT—PCR product. One hundred and twenty-two out of 143 (85.3%) patient samples did not amplify a 1.2kb *hBUB1* RT-PCR product during the first round of *hBUB1*-specific RT-PCR. The cDNA used in the first round of *hBUB1*-specific PCR was synthesized using the Oligo$(dT)_{15}$ primer. Each PCR reaction was performed in duplicate for verification of results. All 122 patient samples with no *hBUB1* RT-PCR product were re-analyzed by another round of PCR for the ubiquitously expressed gene actin. One hundred and sixteen out of 122 (95.1%) patient samples amplified the expected ~500 base pair actin PCR

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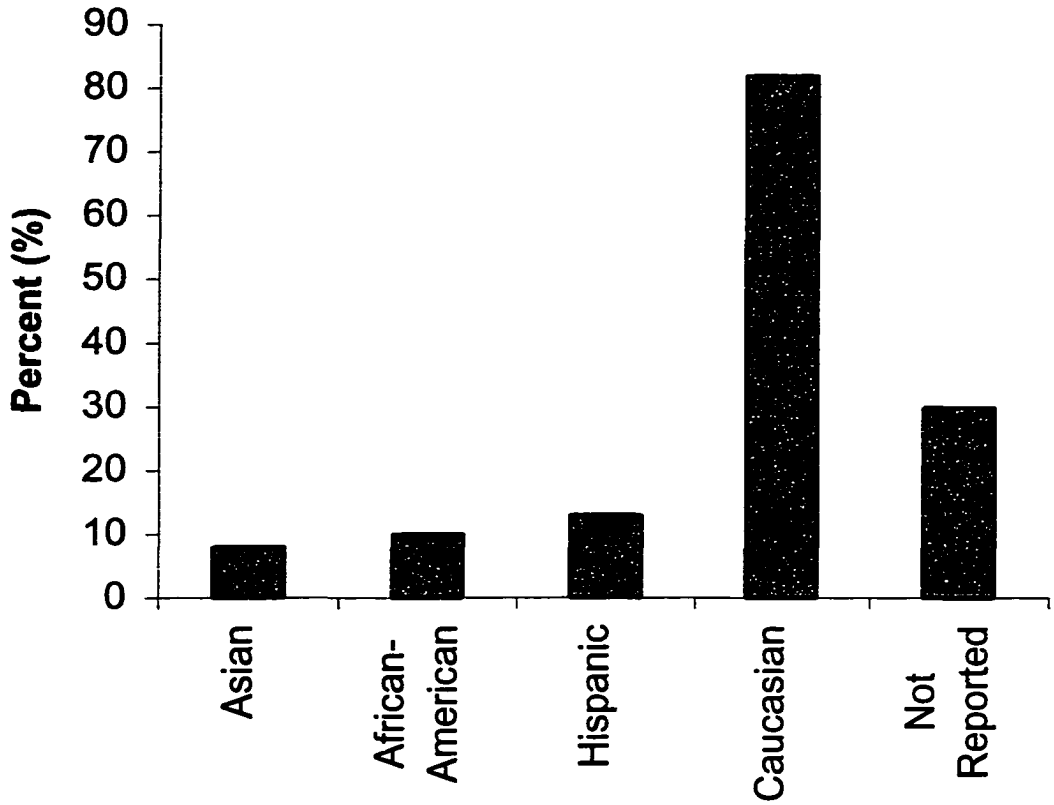

Figure 26. Ethnic Distribution of Patients in Series B

Series B consisted of patients with a variety of bone marrow disorders (Figure 25). Series B samples underwent sequencing of the *hBUB1* RT-PCR product without FISH analysis.

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product. Six patient samples did not amplify any actin PCR product. Therefore, it was assumed that the six patient samples that did not amplify the actin PCR product had degraded RNA. For the other 116 patient samples, the correct amplification of the actin product verified that the RNA was intact and not degraded. All patient samples that showed no *hBUB1* RT-PCR product, but did amplify the expected actin PCR product underwent a second round of cDNA synthesis. In the second cDNA synthesis, the *hBUB1* reverse oligonucleotide PCR primer was used in place of the Oligo$(dT)_{15}$ primer (Figure 27). As stated in Chapter II, the synthesis of new cDNA using the *hBUB1* oligonucleotide reverse PCR primer was performed to increase the likelihood of successful cDNA synthesis of *hBUB1*, provided that the gene was being expressed. The cDNA synthesized using the *hBUB1* oligonucleotide reverse PCR primer was subjected to a new round of *hBUB1*-specific PCR. Twenty-two of the 122 patient samples that underwent the second round of reverse transcription using the *hBUB1* oligonucleotide reverse PCR primer, followed by a second round of *hBUB1* specific PCR, amplified the correct 1.2kb expected *hBUB1* RT-PCR product. These samples were then combined with the patient samples that showed amplification of the expected *hBUB1* RT-PCR product during the first PCR reaction using the Oligo$(dt)_{15}$

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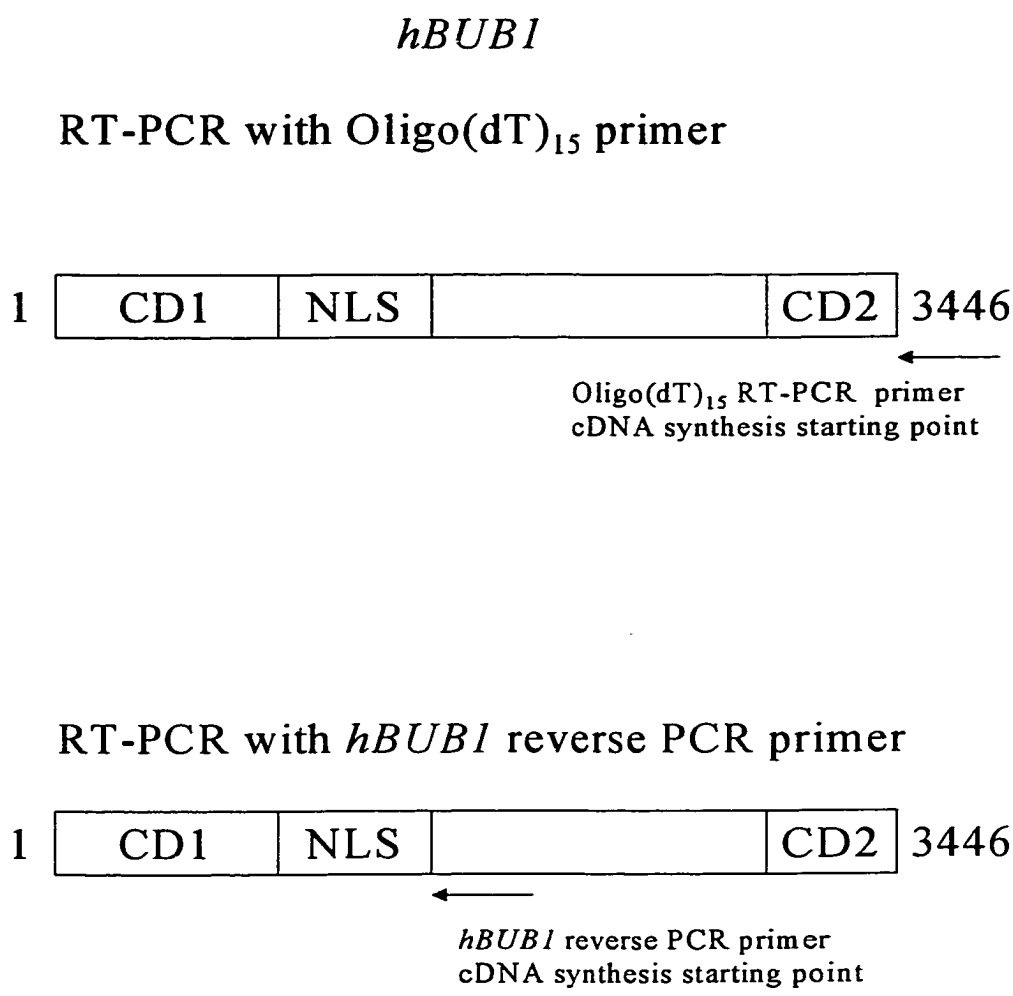

Figure 27. *hBUB1*-Specific RT-PCR Primer Locations

Graphical representation of the location of the RT-PCR primers used in this study. The Oligo(dT)$_{15}$ primer begins cDNA synthesis at the poly A tail, whereas the *hBUB1* reverse PCR primer, when used in reverse transcription in place of the Oligo(dT)$_{15}$ primer, begins cDNA synthesis at the 3′ end of the NLS region in *hBUB1*.

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primer. In total, 43 out of 143 samples (30.1%) amplified the *hBUB1* expected RT-PCR product in this additional patient set (Figure 28). The 30.1% of patient samples from series B that amplified the *hBUB1* RT-PCR product is slightly higher than the 21.7% of patient samples from series A that amplified the *hBUB1* RT-PCR product. Potential reasons for the lack of *hBUB1* RT-PCR amplification are discussed further later in this Chapter.

Sequencing of the *hBUB1* RT-PCR Product in Patient Samples from Series B

Of the 43 patient samples that correctly amplified the 1.2kb *hBUB1* expected RT-PCR product, 14 were subsequently sequenced. All 14 samples underwent one round of reverse transcription, PCR and sequencing. Therefore, these results must be viewed as preliminary. Further sequencing is necessary to increase the confidence interval, and reduce the error introduced by only one sequencing reaction performed for each patient.

Samples from patients 34, 80, 84, 96, 102, 108, 110, 133, 140, 144 and 179 showed no base pair changes present in the CD1 or NLS regions of the *hBUB1* RT-PCR product. Patient 46's sample showed one base pair change at base pair 726; patient 132's sample showed one base pair change at base

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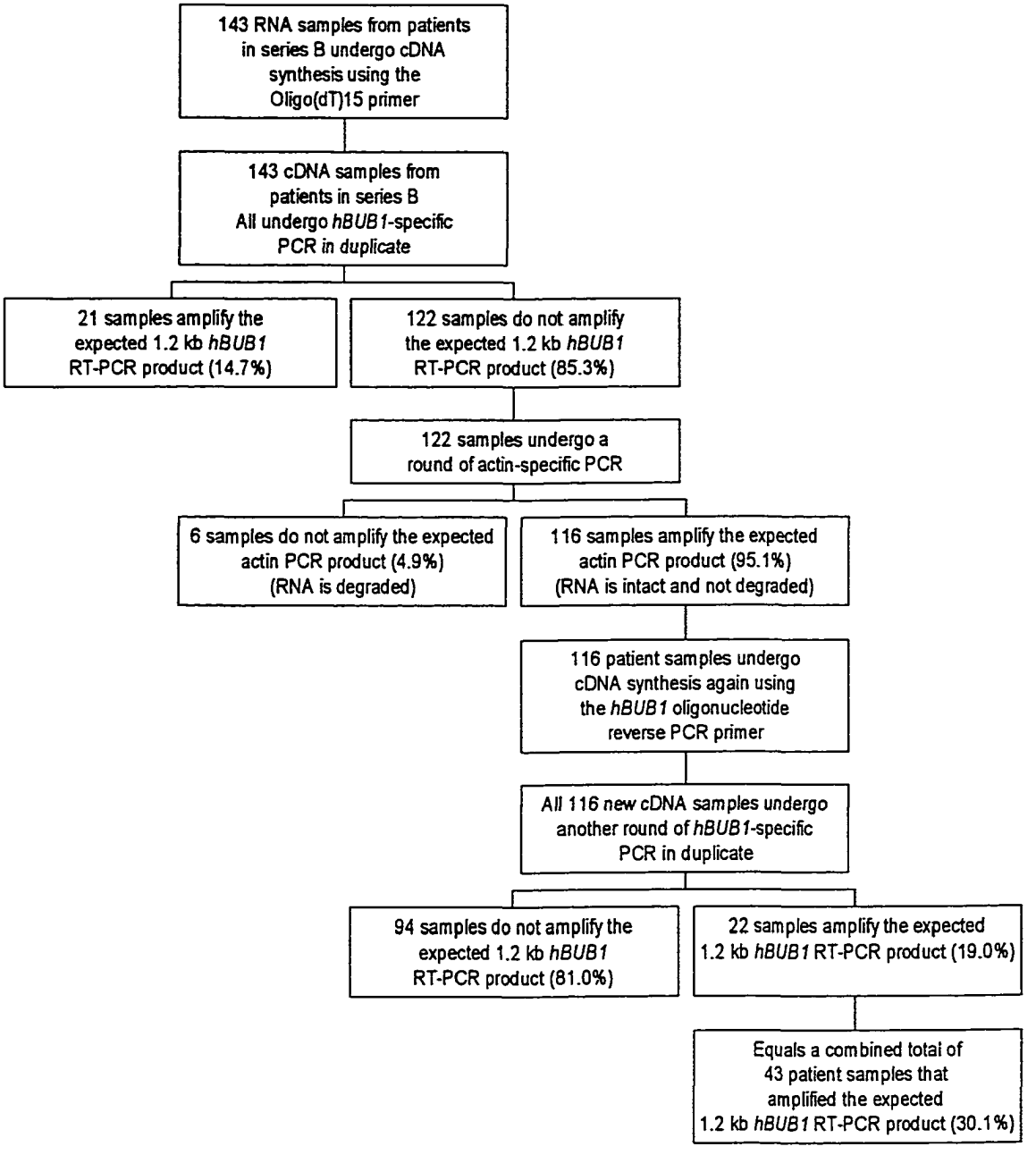

Figure 28. Flow Chart for cDNA Synthesis and *hBUB1*-specific RT-PCR in Patient Samples from Series B

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pair 566, and patient 136's sample showed two base pair changes at base pairs 358 and 731 (Table 5). Again, these base pair changes were not seen in duplicate, and may likely be artifacts. Given the results from the previous extensive sequencing, there was a high frequency of potential artifacts (see end of next paragraph).

Assessment of PCR Conditions

In sequencing patient samples from both series A and series B, I was concerned by the high prevalence of base pair changes in some samples. The normal DNA mutation rate is about one in 10^8 (0.0000001%) base pairs per cell generation (Alberts et al., 1989). Single nucleotide polymorphisms occur in one out of every one to two thousand base pairs (Osgood, personal communication). Therefore, five or six base pair changes in a span of 1214 base pairs is unusually high. In a retrospective analysis, I noticed that PCR reactions performed after August 20, 1999 were completed using a different Taq DNA Polymerase. The protocol after August 20, 1999 utilized a lower grade of Taq Polymerase in the PCR reactions. A low grade Taq Polymerase may have a higher frequency of error when compared to a high quality Taq Polymerase, which has an

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Table 5. Summary of Sequencing Results and Diagnoses for Patient Samples from Series B

Patient #	Diagnosis	BP#	BP Change	Amino Acid Change
34	AA/PRCA			No base pair changes
46	MDS	726	C→T	Ala→Val
80	LGL			No base pair changes
84	MDS			No base pair changes
96	Transformation to Leukemia			No base pair changes
102	MDS			No base pair changes
108	MDS			No base pair changes
110	MDS			No base pair changes
132	Liver Transplantation	566	A→G	Thr→Ala
133	MDS			No base pair changes
136	AA	358 731	T→C A→G	No change Lys→Glu
140	MDS			No base pair changes
144	AA/Hepatitis B			No base pair changes
179	AA			No base pair changes

Comparison of diagnosis for patients in series B to the sequencing results obtained.
Abbreviations: BP=base pair

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error rate of about 3.2 mutations induced per 100,000 base pairs (0.0032%) (Epicentre Technical Support, personal communication). Due to the number of base pair changes in sequences from PCR reactions completed prior to August 20, 1999 when compared to sequences from PCR reactions completed after August 20, 1999, a retrospective analysis was performed to compare these two subgroups.

In looking at the sequencing reactions performed, one can divide the group of sequences into those that showed base pair changes and those that did not show any base pair changes (Table 6). Ignoring patient identifier data, and only looking at the sequences as a whole, it is obvious that a large percentage of the sequences that exhibited one or more base pair changes were completed after August 20, 1999. In addition, the majority of sequences that showed no base pair changes were completed prior to August 20, 1999 (Figure 29). As a final observation, there is an inverse correlation between the number of base pair changes detected and my experience level with respect to sequencing. Therefore, these observations are not likely the result of my inexperience with the sequencing protocols and procedures. The overall number of base pair changes from patient samples in series A was 48 in 40,095 base

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pairs sequenced (0.12%), while samples in series B had 12 base pair changes in 23,085 base pairs sequenced (0.05%).

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Table 6. Comparison Between the Date of the Sequence and the Sequence Result

Sequences Demonstrating No Base Pair Changes	Sequences Demonstrating a Change in 1+ Base Pairs
10/8/99‡	9/5/00‡
8/1/00‡	6/14/99†
8/20/99†	8/1/00‡
8/20/99†	8/17/99†
8/17/99†	9/22/00‡
11/15/00‡	10/26/00‡
6/6/99†	9/19/00‡
10/11/99‡	9/20/00‡
11/01/00‡	9/19/00‡
6/13/99†	9/22/00‡
9/20/00‡	11/15/00‡
2/16/99†	12/6/00‡
6/7/99†	6/16/99†
4/24/99†	6/16/99†
6/7/99†	
4/22/99†	
4/13/00‡	
5/29/99†	
11/15/00‡	
5/30/99†	
5/29/99†	
10/11/99‡	
5/19/99†	

Comparison of sequencing results versus the date sequenced.
† Prior to August 20, 1999
‡ After August 20, 1999

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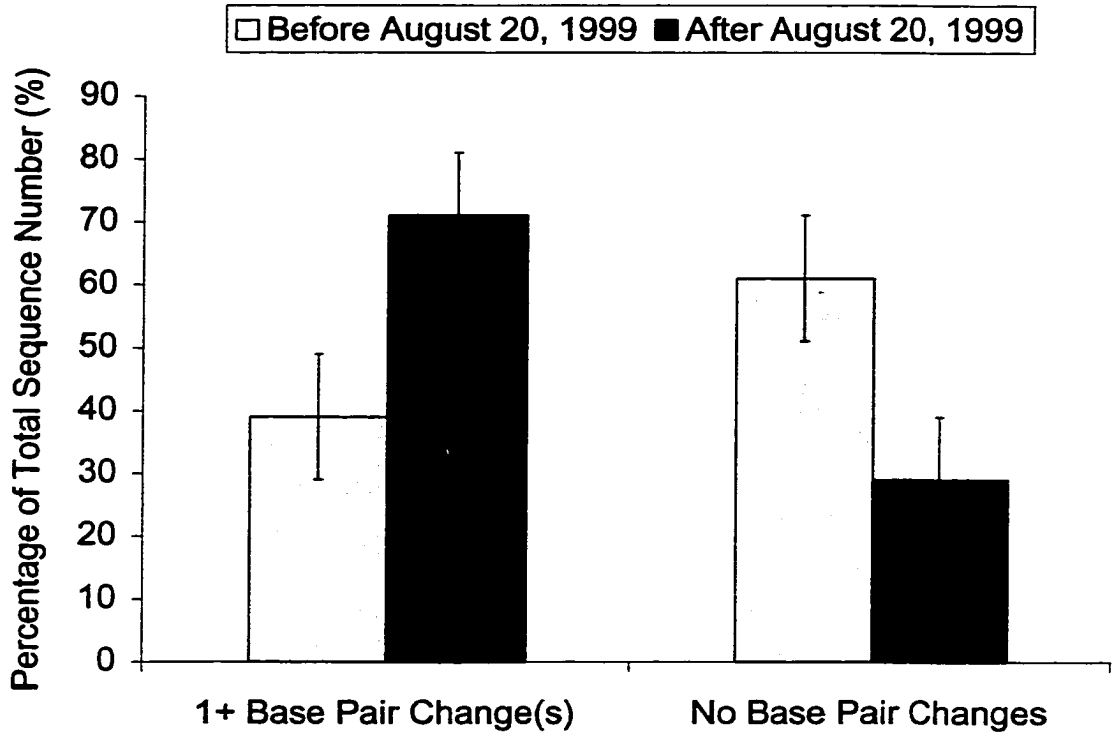

Figure 29. Graphical Representation of the Sequencing Results Versus the Date of Sequencing.

These results indicate that more of the sequences that showed 1+ base pair changes were performed after August 20, 1999. Likewise, more of the sequences that showed no base pair changes were performed prior to August 20, 1999. This indicates that the high rate of base pair changes may be a direct result of the substitution of Taq Polymerase.

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CHAPTER IV

DISCUSSION

NEW INFORMATION ABOUT THE *hBUB1* GENE

Since the inception of this study, many advances have been made with regards to the status of the *hBUB1* gene in various cancers/cancer cell lines. The index cases were reported by Cahill et al., who found two of 19 (10.5%) colorectal cancer cell lines exhibited a mutation within *hBUB1*. The first mutation was a 197 base pair deletion spanning codons 76-141, and the second was a missense mutation (C→A transition at codon 492 resulting in an amino acid change of Tyr→Ser) (Figure 30). Both mutations were found to be heterozygous, with a wild type second allele. When either mutation was expressed in a cell, the cell changed to a CIN phenotype, indicating that the mutations were dominant negative (Cahill et al., 1998). It is important to note that the original colorectal cancer cell lines that exhibited a *hBUB1* mutation all had a CIN phenotype and exhibited high levels of aneuploidy (30-50%) in many different chromosomes, including chromosomes 7 and 8 (Cahill et al., 1998; Lengauer et al., 1997). This level

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of aneuploidy is about 10 times higher than the observed average aneuploidy in this study for chromosomes 7 or 8. In addition, only one patient sample showed an elevated level of aneuploidy in both chromosomes 7 and 8. In contrast, the cell lines examined by Cahill et al., showed consistently high levels of aneuploidy in all chromosomes analyzed (Cahill et al., 1998; Lengauer et al., 1997).

Yamaguchi et al. studied 31 head and neck squamous cell carcinoma and lung cancer cell lines with respect to mutations within *hBUB1*, and found only one base pair change present (T→C transition at codon 220 with no amino acid change) (Figure 30). This implies that mutations in *hBUB1* within head and neck squamous cell carcinoma or lung cancer cell lines are rare, and do not contribute to the high levels of aneuploidy that is characteristic of these cell lines (Yamaguchi et al., 1999).

Imai et al. examined 32 sporadic digestive tract cancers for mutations within the *hBUB1* coding region, and found one heterozygous mutation within *hBUB1* in one rectal cancer case (A→G transition at codon 950 with an amino acid change of Gly→Ser) (Figure 30). This data indicates that mutations within *hBUB1* are not common in the sporadic digestive tract cancers (Imai et al., 1999).

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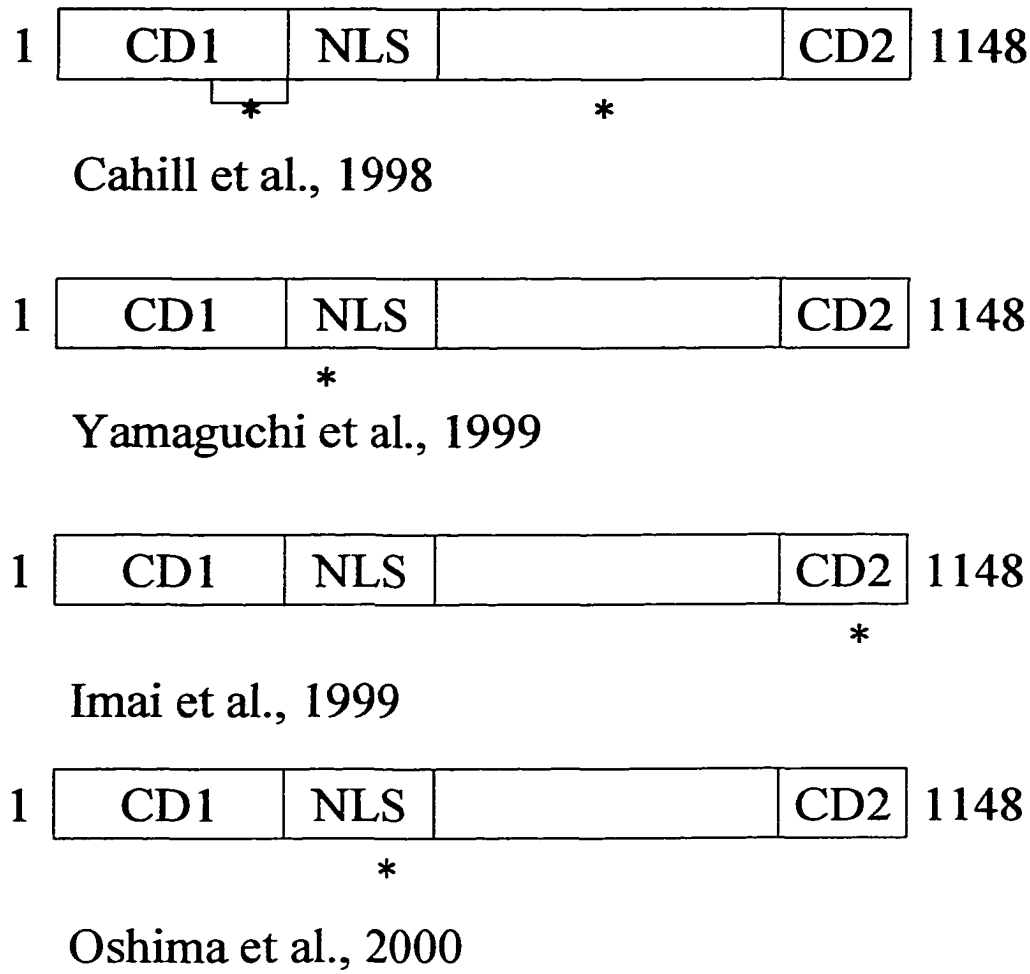

Figure 30. Comparison of Mutation Locations for *hBUB1*.

The four studies that have shown the presence of a mutation within the *hBUB1* coding region are shown here. Each mutation is marked with the * symbol at the approximate codon location that the mutation occurred. None of the mutations occurred in the same location.

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Myrie et al. looked at 19 breast cancer cell lines, all of which were aneuploid, and found no mutations present in the *hBUB1* coding region. Therefore, mutations in *hBUB1* within breast cancer cell lines are a rare event.

Ohshima et al. analyzed ten cases of adult T-cell leukemia and eight cases of B-cell lymphoma for mutations in *hBUB1*, and found one mutation in one of the adult T-cell leukemia patients (G→A transition at codon 250 with an amino acid change of Gly→Asp) (Figure 30). Mutations in *hBUB1* were not common in either adult T-cell leukemia or B-cell lymphoma, and most likely do not contribute to the highly complex karyotypes seen in patients with adult T-cell leukemia (Ohshima et al., 2000).

It is also interesting to note that Jaffrey et al. examined the stability at seven microsatellite markers on chromosome 2q (where *hBUB1* is located) in 32 colorectal cancers and 20 non-small cell lung cancers, both of which exhibit high levels of aneuploidy. The *BUB1* locus was found to be allelically unstable in 62.5% of colorectal cancer samples examined. Instability can result in changes within the coding region, which affects gene transcription. In addition, the *hBUB1* sequence was analyzed in these 32 colorectal cancer tumors, 20 non-small cell lung cancers, and 8 colorectal cancer cell lines, and no mutations were

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present. This indicates that mutations within *hBUB1* are most likely not responsible for the high levels of aneuploidy that is commonly seen in colorectal cancer tumors, non-small cell lung cancers and colorectal cancer cell lines (Jaffrey et al., 2000).

In combination, the data summarized above indicate that mutations in the *hBUB1* coding region in a variety of cancers and cancer cell lines are very rare occurrences. The information from this study is in agreement with the above data, and showed that a mutation within *hBUB1* in samples from patients with aplastic anemia is also a rare occurrance.

FISH RESULTS

The presence of clonal cytogenetic abnormalities in patients with AA is evidence of a pre-leukemic state (Marsh and Geary, 1991). Some researchers have shown that cytogenetic analysis by FISH is more sensitive than karyotype analysis (Thurston et al., 1999; Socié et al., 2000; Young, 1999; Young, 2000). Therefore, FISH may detect early clonal cytogenetic abnormalities normally missed by a karyotype analysis. Since monosomy 7 and trisomy 8 are common in MDS and ANLL (Socié et al., 2000),

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chromosomes 7 and 8 were chosen for FISH analysis in this study. Hypothetically, the presence of chromosomal aberrations may indicate a higher likelihood of later transformation to MDS or ANLL. These aberrations may be easier to detect using FISH.

The statistical analyses on the results obtained by FISH analysis on bone marrow samples from patients in series A (AA patients only), showed no statistically significant difference between the patient samples as a whole versus control samples with respect to the level of aneuploidy for either chromosome 7 or 8. There were certain patient samples that individually showed increased levels of aneuploidy in chromosome 7, chromosome 8 or a combination of both chromosomes. Seven of 46 patient samples (15.2%) showed increased levels of aneuploidy. Four of the seven patient samples (57.1%) showed newly detected aneuploidy that was previously unreported in a karyotype analysis. FISH may be useful in detecting chromosomal abnormalities earlier within the disease progression. In this study, FISH results were more than twice as sensitive in detecting cytogenetic abnormalities when compared to the results obtained by a karyotype analysis.

The level of aneuploidy in patient samples that showed statistically significant levels of aneuploidy ranged from

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3.33% to 12.07%. These values are relatively low when compared to literature values for aneuploidy levels found in colorectal cancer cell lines (30-50%). In addition, values reported by Lengauer et al. demonstrated a high level of aneuploidy in all chromosomes assayed, not selective aneuploidy in just one or two chromosomes. "CIN affects all chromosomes equally" (Lengauer et al., 1997), however, of the seven patient samples showing statistically significantly elevated levels of aneuploidy, only one patient sample showed an increased level of aneuploidy for both chromosomes 7 and 8. The remaining six patient samples showed selective aneuploidy in only one of the two chromosomes assayed.

The mitotic index, obtained to estimate the cell growth rate, was uniformly low and not statistically significantly different in a 2-sample t-test comparison of patient samples versus control samples ($p=0.571$). The patient samples in this study do not appear to be rapidly dividing, but neither do the control bone marrow samples. Cahill et al. (1998) demonstrated that the mitotic index was significantly reduced in cells that expressed a mutant *hBUB1*. The cells with wild-type *hBUB1* showed a mitotic index that was 90% of the control value, while cells with a mutant *hBUB1* showed a mitotic index that was 60% of the

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control value (Cahill et al., 1998). In comparison, the data from this study (series A patient samples only) indicated that the average patient MI was 0.44%, while the average control MI was 0.33%. These data are different from the data previously reported by Cahill et al. (1998). However, in the study by Cahill et al. (1998), pure colorectal cancer cell lines were used, compared to the cells in this study, which were taken from patients with decreased bone marrow. The low mitotic index may be of importance when evaluating the *hBUB1*-specific RT-PCR results from the patient samples in this study.

RT-PCR RESULTS

The absence of *hBUB1*-specific RT-PCR products in patient samples from this study may have occurred as a direct result of low cell regeneration. In some samples, the intensity of the expected RT-PCR band varied, indicating that the level of *hBUB1* expression may vary from patient to patient. DNA microarray assays have detected that in yeast, *BUB1* peaks during the G1 phase of the cell cycle (Spellman et al., 1998). Perhaps those patients who did not amplify any *hBUB1* RT-PCR product had a very low level of *hBUB1* expression, or had the majority of the cells not

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in the G1 phase of the cell cycle, so that the level of *hBUB1* was too low to be detected by conventional RT-PCR methods. The same concentration of RNA was used in all of the cDNA synthesis reactions, however different volumes of RNA were used in each reaction, depending on the concentration of the RNA. RT-PCR was successful in amplifying the *hBUB1*-specific RT-PCR product in samples with an RNA concentration as low as 0.062µg/µl. On the contrary, no *hBUB1*-specific RT-PCR product was detected in samples with an RNA concentration as high as 4.440µg/µl.

If *hBUB1* were expressed in dividing cells, one would expect successful amplification of the *hBUB1* RT-PCR product in all dividing cells. However, only a small portion of the patients assayed exhibited a RT-PCR product (21.7% of patient samples in series A and 30.1% of patient samples in series B), and only 60% of the control samples amplified the correct *hBUB1*-specific RT-PCR product. Since part of the PCR verification included the synthesis of the complimentary DNA using a *hBUB1* oligonucleotide reverse PCR primer, the lack of amplification was most likely not due to incomplete cDNA synthesis. However, a complex secondary structure could have interfered with the cDNA synthesis since the samples were not heated to eliminate secondary structure prior to the reverse transcription reaction.

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Another explanation could be the presence of hypermethylation within the *hBUB1* promoter region, which may inhibit the expression of the gene. The presence of an actin RT-PCR product verifies that the overall RT-PCR reaction was successful. In addition, more control bone marrow samples need to be assayed to obtain a more accurate percentage of *hBUB1*-specific RT-PCR amplification. The investigation of the low amplification rate is deserving of further research.

SEQUENCING RESULTS

If aneuploidy were caused by a mutation within a mitotic spindle checkpoint gene, *hBUB1* would be a likely candidate based on early literature. Since hBUB1 is required for the spindle checkpoint within the cell cycle, the lack of hBUB1 function could hypothetically lead to the demise of the spindle checkpoint and premature entrance into anaphase before all chromosomes are properly aligned on the metaphase plate. It is also the most characterized of the group of genes which comprises the mitotic spindle checkpoint.

However, if a mutation in *hBUB1* did abrogate the function of the mitotic spindle checkpoint, one would expect to see

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consistent levels of aneuploidy in all chromosomes. It is highly unlikely that a mutation in *hBUB1* would target a specific chromosome, thereby enabling chromosome-specific aneuploidy to occur. In this study, aneuploidy levels for both chromosomes were not consistent.

With respect to the sequencing results obtained by this study, it is reasonable to conclude that the base pair changes seen after August 20, 1999 are not real, but are due to artifacts introduced at the PCR stage of the experiment. Using this assumption, I modified the presentation of the sequencing results (Table 7).

In Table 7, I assumed that all base pair changes found in patient samples analyzed after August 20, 1999 were artifacts. In addition, I have removed all base pair changes that did not result in an amino acid change (as this would probably not affect the function of the protein). This greatly reduces the number of potential mutations due to actual base pair changes.

The sample from patient #43 (adjusted) shows the presence of a base pair change at base pair 845, which is located within the NLS coding region (Cahill et al., 1999). FISH analysis failed to detect any significant amount of aneuploidy for either chromosome 7 or 8 for this patient. Therefore, it is reasonable to conclude that the sample

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Table 7. Sequencing Results Adjusted for Taq-induced PCR Artifact

Patient #	BP#	BP Change	Amino Acid Change
43	845	G→A	Glu→Lys
82	632	G→A	Gly→Arg
	908	C→T	Gln→stop
132	566	A→G	Thr→Ala
136	731	A→G	Lys→Glu

Corrected sequence results for patient samples in series A and series B. All samples listed were sequenced prior to August 20, 1999 and had a base pair change that resulted in an amino acid change. Note that none of the above sequencing results were confirmable.
Abbreviation: BP=base pair

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from patient 43 either 1) had no mutations within the CD1 or NLS regions of *hBUB1* (existing base pair change is an artifact), 2) the base pair change present does not alter the function of the BUB1 protein, or 3) *hBUB1* does not play a direct role in the creation of aneuploidy.

The sample from patient #82 shows two base pair changes, located at base pairs 632 and 908, both of which are located in the NLS region. If the base pair change at number 908 were real, it would induce the formation of a stop codon. This would lead to the translation of a truncated protein, which may be unable to function. A mutation in the NLS region may affect the ability of the BUB1 protein to localize to the nucleus. Since BUB1 is active at the kinetochore, it must be able to travel into the nucleus from the cytoplasm to be able to function. In this patient sample, statistically significantly elevated levels of aneuploidy were not observed for either chromosome 7 or 8. In addition, the majority of the sequence data obtained for the sample from patient 82 showed no base pair changes present. It is therefore reasonable to conclude that due to the high probability of Taq-induced PCR artifact and because this patient did not exhibit any signs of a loss of the mitotic spindle

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checkpoint (the appearance of aneuploidy), the base pair changes seen are most likely artifactual.

Samples from patients #132 and #136 did not undergo FISH analysis. Regardless, it is possible to draw similar conclusions with respect to the sequencing results from these patient samples, and assume that the base pair changes seen are most likely the result of Taq-induced PCR artifact.

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CHAPTER V

CONCLUSIONS

1. New FISH data on AA patient samples

This study showed that aneuploidy in patients with aplastic anemia is a rare event, with only 7 of 46 (15.2%) patient samples displaying aneuploidy for chromosome 7 and/or 8. It is of importance to note that the percent occurrence may not be synonymous with the percent of new cytogenetic events, as clonal selection may play a role in the expansion of a cell type. It is possible that the aneuploidy detected represents early clonal selection for the apparent cytogenetic abnormalities, which may increase over time.

2. FISH was approximately twice as sensitive as conventional cytogenetic analysis by karyotype

In this study, FISH detected more than twice as many cytogenetic abnormalities than the karyotype analysis. FISH may be beneficial in detecting early cytogenetic abnormalities.

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3. **African-American males showed a statistically significantly elevated level of monosomy 8**

In a multivariate ANOVA statistical analysis, African-American males showed a greater likelihood of exhibiting monosomy 8 ($p=0.016$). These results should be viewed with caution due to the low sample size number, but may be beneficial in the future if perhaps African-American males have a genetic variant making them more susceptible to developing AA.

4. **One patient showed statistically significantly elevated levels of aneuploidy for both chromosomes 7 and 8**

One patient sample exhibited high levels of aneuploidy for both chromosomes 7 and 8, which is more characteristic of a CIN phenotype. However, this appears to be a rare occurrence within patients with AA.

5. **Two patients showed the presence of an unusual insertion within the forward primer area of *hBUB1***

Two patients (#42 and #52) exhibited an insertion within the forward primer area in *hBUB1*, which would be predicted to inhibit the complete translation of the protein product. If verified, these insertions would suggest a loss of normal hBUB1 function, and perhaps an abrogated mitotic

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spindle checkpoint. Verification is needed to ensure that the insertions are not due to faulty ligation with the plasmid vector or errors with the polymerase activity. The sample from patient #52 did show a statistically significantly elevated of aneuploidy, however the aneuploidy does not appear to be due to the insertion, as it would likely been seen in both patient samples.

6. **Preliminary sequencing results indicated that base pair changes in *hBUB1* within AA patients are rare occurrences**

The majority of patient samples did not exhibit any true base pair changes within the *hBUB1* CD1 and NLS regions. This correlates to recent data from other laboratories showing that mutations in *hBUB1* in a variety of cancers and cancer cell lines rarely occur. This suggests that the aneuploidy seen in these patient samples and in other studies may not be due to a mutation within the mitotic spindle checkpoint gene *hBUB1*. However, there are many possible causes of aneuploidy, with mutations in a mitotic spindle checkpoint gene being only one of the different pathways. Further investigation and verification is required to establish a more concrete conclusion.

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APPENDIX

PATIENT DATA

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ID #	Diagnosis	Sex	Race	Cytogenetics
17	MDS	F	W	46,xx
18	MDS	M	W	46,xy,del(20)(q11.2q13.3)[4]/46,xy[19]
21	MDS/5q-	F	W	46,xx,del(5)(q22q33)[10]/46,xx[10]
23	5q-	F	W	46,xx,del(5)(q13q33)[17]/47,xx,t8[3]
25	AA	M	W	46,xy
26	Thrombocytosis	M	W	46,xy,del(17)t(?13;17)(q14;p11,2)[13]/46,xy[8]
27	MDS	M		46,xy,-7[14]/46,xy[16]
28	MDS	M	W	46,xy
29	AA	M	H	46,xy
30	AA	F	H	insufficient
31	AA	M	A	46,xy
32	AA	M		46,xy
33	AA	M	H	46,xy
34	AA/PRCA	M	W	46,xy[3]
35	AA	M	W	46,xy[30]
36	MDS	M	W	46,xy[30]
37	AA	M	W	46,xy[29]
38	MDS/5q-	F		46,xx,del(5)(q15q35)[3]/46,xx[3]
39	AA	M	A	46,xy
40	AA	F	W	46,xx[31]
41	AA	F	H	46,xx
42	AA	M	B	46,xy[30]
43	AA/Porvovirus	F	W	46,xx[30]
44	RA/5q-	F	W	46,xx,del(5)(q15q35.1)[20]
45	MDS	M	W	46,xy
46	MDS	F		46,xx[30]
47	MDS	M	W	46,xy[30]
48	MDS	M	W	46,xy
49	Porvovirus	F		46,xx
50	AA/Trisomy 8	F	W	TRI8
51	AA	F		46,xx
52	AA/Trisomy 8	M	W	47,xy,+8[13]/46,xy[7]
53	MDS	M	W	insufficient
54	MDS	M	W	46,xy
55	AA/PNH	F	W	46,xx,inv(9)(p11q13)c
56	MDS	M	W	46,xy[30]
57	AA	M	W	46,xy
58	AA	M	A	46,xy
59	AA	M	A	insufficient
60	AA	F	W	46,x,ins(x;1)(p11.4;q21q42.3)[9]/46,xx[41]
61	5q-	M	W	46,xy,del(5)(q31q35),inv(9)(p11.2q13)[8]/46,xy,inv(9)(p11.2q13)[13]
62	MDS	M	W	46,xy[30]
63	MDS	M	A	46,xy
64	AA	F	H	46,xx[30]
65	AA	F	H	46,xx[30]
66	RAEB	M	W	47,xy,+21[3]/48,iden,+13[2]/46,xy[17]

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ID #	Diagnosis	Sex	Race	Cytogenetics
67	PRCA	F	W	46,xx
68	MDS/RAEB	M	W	46,xy[30]
69	AA	M	H	46,xy
70	AA/Hepatitis C	M	W	46,xy[30]
71	Pancytopenia	M	W	46,xy,del(20)(q11.2q13)[12]/46,xy[10]
72	PNH	M		46,xy
73	AA	F		46,xx[30]
74	MAA/PNH	M	H	46,xy,del(20)(q11.2q13.3)[2]/46,xy[31]
75	Myeloproliferative Disease	M	W	46,xy,del(20)(q11.2q13.3)[5]/46,xy[15]
76	Neutropenia	F	W	46,xx[30]
77	Pancytopenia/AA	F	W	46,xx[30]
78	RA/5q-	F	W	46,xx,del(5)(q22q33)[12]/46,xx[2]
79	MDS	M	B	46,xy
80	LGL	M	W	46,xy
81	AA	M	B	46,xy
82	AA	M	H	46,y,t(x;8)(p22.1;q24.1)
83	Moderate AA	F	W	46,xx[30]
84	MDS	M	W	46,xy,-7,-15,-17[1]/46,xy[49]
85	AA/PNH	F	W	46,xx
86	AA	M	H	46,xy
87	SAA	F	B	46,xx
88	SAA	M	W	46,xy[30]
89	AA	F	W	46,xx
90	Moderate AA	F	H	46,xx
91	MDS	M	W	46,xy,del(11)(q23q25)[14]/46,xy,dup(1)q(44q12),del(17)(p11.2p12)[4]/46,xy[2]
92	MDS	F	W	46,xx
93	MDS	M	W	46,xy
94	Pancytopenia post acute Promeylocytic leukemia	M	W	46,xy,del(20)(q11.2q13.3)[16]/47,idem,+8[3]/46,xy[1]
95	AA	F	W	46,xx
96	Probable progression to leukemia	M	W	46,xy
97	MDS	M		
98	AA	F	B	46,xx[30]
99	AA/Trisomy 8	M	W	47.xy,+8[14]/46,xy[7]
100	MDS	F	A	
101	AA/PNH/LGL	M	B	45,x,-y[14]/46,xy[6]
102	MDS	M	W	46,xy[30]
103	MDS	M	W	46,xy[27]
105	AA	F	W	46,xx[30]
106	AA/PNH	M	A	46,xy
107	MDS	F	W	46,xx
108	MDS	M	W	46,xy[30]
109	MDS	M	B	46,xy,del(11)(q21q23)[10]/46,xy[10]
110	MDS	F		46,xx[30]
111	AA	M		46,xy[30]
112	Anemia	M	W	46,xy

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ID #	Diagnosis	Sex	Race	Cytogenetics
113	Moderate AA	F	W	46,xx
114	MDS	F	W	insufficient
115	AA	F	W	46,xx
116	AA	F	A	46,xx[30]
117	AA	F	H	46,xx[30]
118	MDS	M	W	
119	AA	M		46,xy,inv(9)(p11q13)c
120	MDS	F		46,xx
121	AA	M	W	46,xy
122	AA	M		
123	Myeloproliferative disease	M		46,xy,t(5:12)(q33;p13)[7]/46,xy[13]
124	PNH	M	B	
125	PRCA	M	W	
126	MDS	M	H	47,xy,+8[7]/46,xy[13]
127	Thrombocytopenia	F		46,xx
128	AA	F	W	46,xx[30]
129	AA	F	W	46,xx[30]
130	AA/PNH	F	W	46,xx
131	MDS	M	W	
132	Liver Transplant	F	H	
133	MDS	M	W	
134	new MDS	M	W	
135	AA	M	H	
136	AA	F	W	
137	AA	F	W	46,xx[30]
138	Anemia	M	B	
139	AA	F		46,xx
140	MDS	F	W	
141	AA	M	W	
142	AA	F	W	46,xx[30]
143	MDS	M	W	
144	AA Hepatitis/aplasia	M	B	
145	AA v MDS	F	A	
146	AA v MDS	M	W	46,xy[21]
147	AA	M	W	46,xy[30]
148	AA	M	W	
149	MDS	M	W	
150	RAEB	M		
151	PRCA	M		
152	Thrombocytopenia	M	W	
153	AA/PNH	F	W	46,xx
154	PNH		W	
155	MDS	M	W	
156	MDS	M	W	
157	AA	M	W	46,xy[30]

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ID #	Diagnosis	Sex	Race	Cytogenetics
158	AA	F	W	
159	MDS	F	W	
160	MDS			
161	Pancytopenia	F	W	
162	AA		A	
163	AA	F	W	
164	Acute Pancytopenia	F	W	
165	AA	M	W	
166	AA	M	W	46,xy[30]
167	AA	F		48,xxxc,+8[5]/47,xxxc[15]
168	AA	F		trisomy 8
169	AA	M	H	46,xy
170	PNH	F		
171	MDS		H	
172	MDS	M		
173	PNH	M	W	
174	Hypoplastic Anemia	M	A	
175	AA	M	H	
176	AA	M	B	
177	PNH	F	B	
178	RA	F	W	
179	AA	F	B	
180	AA	F	H	
181	AA	M	H	
182	AA	F	W	
183	AA	F		
184	MDS	M	W	
185	AA	F		
186	MDS	M	W	
188	Pancytopenia	M	W	
190	Pancytopenia	F	W	
191	AA	M	W	
192	AA	M	B	
193	Pancytopenia	F	W	
194	RA	F		
195	neutropenia		W	
196	MDS	M		
197	PNH/AA	F	W	
198	MDS	M	W	
199	MDS	M	W	
200	new MDS	F		
201	AA	M	A	
202	AA	M	B	
203	MDS	F	W	
204	AA	F		

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ID #	Diagnosis	Sex	Race	Cytogenetics
205	AA	M	H	
206	MDS	F	W	
207	AA	M		
208	AA			
209	AA	M	H	
210	Pancytopenia	M		
211	AAvMDS (RA)	M		
212	AA	F	W	

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ID #	BUB	Actin	R-cDNA	7(1)	7(2)	7(3)	8(1)	8(2)	8(3)	Treatment
17	no	+	-							new; evaluate for ATG
18	no	+	-							new, evaluate for ATG
21	no	+	-							1 yr f/u non-responsive to ATG
23	no	+	-							failed ATG
25	no	+	-							new patient. No treatment
26	no	+	-							
27	1.2	+	+							new patient. No treatment
28	no	+	-							EPO/G-CSF nonrespomder 1 yr ago
29	no	n/a	n/a	4	294	2	2	295	3	Cytoxan/CSA responder
30	no	+	-							post liver transplant. No treatment
31	no	+	-	4	296	0	10	289	1	no treatment
32	no	n/a	n/a							Danzol recently
33	no	n/a	n/a	3	296	1	11	289	0	ATG/CSA Relapased on CSA
34	1.2	n/a	n/a							None?
35	no	+	-							Treated with ATG & CSA
36	1.2	+	+							6mo f/u Post ATG non-responder
37	no	+	-	9	291	0	7	292	1	6mo f/u Post cytotoxin?/CSA Responder
38	no	+	-							1 yr f/u failed ATG
39	no	n/a	n/a	2	297	1	7	289	4	5 yr f/u post ATG/CSA responder
40	no	+	-	10	287	3	7	291	2	6 yr f/u on CSA post ATG/CSA relapsed
41	no	n/a	n/a	3	295	1	5	293	2	CSA platelet fx dependent?, ATG, IL-3, Neupogem?
42	1.2	+	+	8	292	0	4	295	1	4 years post ATG/CSA responder
43	1.2	+	+	9	289	2	7	290	3	ATG no response
44	no	+	-							no treatment
45	1.2	n/a	n/a							new
46	1.2	+	+							new
47	no	+	-							new ?- NR
48	no	+	-							new
49	no	+	-							none
50	no	+	+							ATG/CSA, on ? CSA
51	no	+	-							ATG/CSA responder
52	1.2	+	+	8	290	2	1	238	61	ATG/CSA responder now relapsed
53	no	+	-							ATG/CSA responder
54	no	+	-							ATG/CSA non-responder
55	no	+	-							ATG/CSA responder
56	no	+	-							Growth factor non responder
57	no	+	-	7	292	1	4	293	3	ATG,CSA.De??responder
58	no	+	-	6	294	0	16	284	0	no treatment
59	no	+	-							no treatment
60	no	+	-	5	295	0	9	289	2	2yr post ATG/CSA respomder
61	no	+	-							Failed ATG
62	no	+	-							Failed 5-AZA Failed Neumega?
63	no	+	-							never treated
64	no	+	-	4	295	1	6	292	2	ATG/CSA 1yr post tr. On CSA relapsed
65	no	+	-							ATG/CSA responder 1yr
66	no	+	-							None

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ID #	BUB	Actin	R-cDNA	7 (1)	7 (2)	7 (3)	8 (1)	8 (2)	8 (3)	Treatment
67	no	+	-							1yr post ATG
68	no	+	-							None
69	no	+	-							6mo post cyotoxin?/CSA
70	no	neg	-							yr f/u ??
71	no	+	-							None
72	1.2	+	+							None
73	no	+	-							ATG/CSA Relapsed
74	no	neg	-							ATG Responder
75	no	+	-							None
76	no	+	-							None
77	no	+	-	5	295	0	4	294	2	CSA and Prednisone & EPO
78	no	+	-							transient responder to ATG, s/p CSA --> NR
79	no	neg	-							ATG responder then relapse, retreat ATG --> normal counts
80	1.2	n/a	n/a							None
81	no	+	-	7	293	0	7	291	2	None
82	1.2	n/a	n/a	9	290	1	4	292	4	6 months post ATG/CSA
83	no	+	-							None
84	1.2	n/a	n/a							ATG
85	no	+	-	3	295	2	7	284	8	CSA
86	no	+	-	2	297	1	5	294	0	1 year post cytoxen, 2 years post ATG
87	no	+	-	7	293	0	11	284	5	ATG/CSA 6 mo post tx, non responder
88	no	+	-	6	294	0	6	290	4	ATG/CSA 6 mo post tx, responder
89	no	+	-							ATG/CSA, Davarol, non responder
90	1.2	n/a	n/a	5	293	2	0	297	3	none
91	no	n/a	n/a							1.0yr F/U ATG,
92	no	+	-							never treated
93	no	+	-							never treated
94	no	neg	-							chemotherapy
95	no	+	-	5	294	1	1	298	1	18mo post ATG/CSA responder
96	1.2	n/a	n/a							none
97	no	neg	-							none
98	no	+	-	7	292	1	4	289	7	1year post cytoxen/CSA
99	no	+	-	2	298	0	8	204	88	1 1/2 year post ATG/CSA
100	no	neg	-							nonresponder 10/95
101	no	+	-	8	292	0	10	286	4	6mo post ATG/CSA non responder
102	1.2	n/a	n/a							6mo f/u non responder ATG
103	no	+	-							failed - new MDS
105	no	+	-							ATG/CSA responder - now cts dropping
106	no	+	-							no treatment
107	no	+	-							no treatment - to start CSA today
108	1.2	n/a	n/a							no treatment
109	no	+	-							failed ATG 1 year f/u
110	1.2	n/a	n/a							none
111	no	+	-	12	286	2	13	279	8	none
112	no	+	-							none

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ID #	BUB	Actin	R-cDNA	7 (1)	7 (2)	7 (3)	8 (1)	8 (2)	8 (3)	Treatment
113	no	+	-	3	254	2	7	251	1	none
114	no	+	-							multiple Amifostine etc
115	no	+	-	3	296	1	4	292	4	ATG/CSA 1yr post tr. Responder, relapse now?
116	no	+	-	8	289	3	3	287	10	not treated
117	1.2	n/a	n/a	8	292	0	9	288	3	ATG/CSA relapsed currently on CSA
118	1.2	n/a	n/a							never treated
119	no	+	-							None
120	1.2	n/a	n/a							Untreated
121	1.2	n/a	n/a	7	293	0	5	294	1	ATG/CSA 6 mo ago, responder
122	no	+	-							1 yr ago Cytoken/CSA, responder
123	no	+	-							none
124	1.2	+	+							new patient. No treatment
125	1.2	+	+							new patient. No treatment
126	1.2	+	+							new patient. No treatment
127	no	+	-							none
128	no	+	-	6	110	0	14	100	2	ATG, IVIG, Steroids, spleenectomy
129	1.2	+	+	2	296	1	0	299	0	none
130	no	+	-	4	161	0	2	163	0	ATG/CSA responder
131	no	+	-							EPO
132	1.2	n/a	n/a							ATG 6mo post on FK506
133	1.2	n/a	n/a							6mo f/u CSA
134	1.2	+	+							EPO
135	1.2	+	+							Relapsed 1 1/2 years non-responder, ATG/CSAx2
136	1.2	n/a	n/a							ATG/CSA relapsed, CSA dependent
137	1.2	n/a	n/a	7	292	1	6	287	7	none
138	1.2	+	+							none
139	no	+	-	1	299	0	2	297	1	ATG/CSA Relapsed on CSA
140	1.2	n/a	n/a							new patient. No treatment
141	1.2	n/a	n/a							6yr, post ATG/CSA
142	no	+	-	1	298	1	8	292	0	new patient. No treatment
143	no	+	-							Untreated
144	1.2	+	+							Cytoxan/CSA I yr post treatment Responder
145	no	+	-							None
146	no	+	-	4	295	1	3	291	6	new patient. No treatment
147	no	+	-	4	296	0	8	290	2	new patient. No treatment
148	1.2	+	+							new patient. No treatment
149	1.2	+	+							new patient. No treatment
150	no	+	-							ATG non-responder
151	no	+	-							new patient. No treatment
152	no	+	-							new patient. No treatment
153	1.2	+	+	1	297	2	8	291	1	new patient. No treatment
154	no	+	-							new patient. No treatment
155	1.2	+	+							6 mo f/u CSA responder
156	1.2	n/a	n/a							EPO
157	no	+	-	3	297	0	6	294	0	1yr post cytoxen/csa, 6mo post ATG/CSA, no response for both

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ID #	BUB	Actin	R-cDNA	7(1)	7(2)	7(3)	8(1)	8(2)	8(3)	Treatment
158	1.2	+	+							11 years post treatment
159	no	+	-							none
160	no	+	-							none
161	no	+	-							7 years, none
162	no	+	-							ATG/CSA non-responder
163	no	+	-							ATG/CSA on ATG
164	no	+	-							N Bupogen
165	no	+	-							ATG/CSA 8 years post responder, hepatitis C
166	no	+	-	9	290	1	4	296	0	new patient, no treatment
167	no	+	-	5	295	0	8	291	1	ATG/Decadurobulie??
168	no	+	-	5	295	0	12	257	31	ATG/CSA
169	no	+	-	2	298	0	3	294	3	ATG/CSA, cytotoxin
170	1.2	n/a	n/a							none
171	no	+	-							new patient, no treatment
172	no	+	-							new patient, no treatment
173	1.2	+	+							none
174	no	+	-							none
175	no	+	-							1 year post Cytoxan/CSA
176	no	n/a	n/a							ATG/CSA x2
177	1.2	+	+							none
178	1.2	+	+							none
179	1.2	+	+							ATG/CSA non-responder
180	1.2	+	+							ATG/CSA x3 approx 1 year from ATG responder
181	no	+	-							none
182	no	+	-							ATG/CSA
183	1.2	+	+							Cytotoxin non-responder
184	no	+	-							none
185	no	+	-							ATG/CSA
186	no	+	-							none
188	no	+	-							none
190	no	n/a	n/a							none
191	1.2	n/a	n/a							ATG/cSA 1 year post treatment
192	no	+	-							none
193	1.2	n/a	n/a							none
194	no	+	-							
195	no	+	-							
196	1.2	n/a	n/a							
197	no	+	-							
198	no	+	-							ATG failed
199	no	+	-							ATG failed
200	no	+	-							None
201	no	+	-							6 yrs post ATG/CSA Responder
202	1.2	+	+							Cytoxan?CSA 6.5mo response?
203	no	+	-							None
204	no	+	-							ATG/CSA

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ID #	BUB	Actin	R-cDNA	7 (1)	7 (2)	7 (3)	8 (1)	8 (2)	8 (3)	Treatment
205	1.2	n/a	n/a							
206	no	+	-							
207	no	+	-							
208	no	+	-							
209	no	+	-							
210	no	+	-							
211	no	n/a	n/a							
212	no	+	-							ATG/CSA

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UNIVERSITY EDUCATION

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May 1997-December 1998: Research Assistant, Center for Pediatric Research, Eastern Virginia Medical School and Old Dominion University

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PUBLICATIONS

Hynes W.L., Dixon A.R., Walton S.L., and **Aridgides L.J.** (2000). The extracellular hyaluronidase gene (hylA) of Streptococcus pyogenes. FEMS Microbiol Lett *184(1)*, 109-12.

Aridgides, L.J., Stacey, M., Brihn, L., Scott, D., Osgood, C., and Kearns, W.G. (2001). Fluorescence in situ hybridization on fresh bone marrow cells and sperm using alkaline denaturation. Biotechniques. (Submitted).

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