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13. ABSTRACT *(Maximum 200 words)* Dose intensive anthracycline-based adjuvant regimens improve disease-free survival in breast cancer patients; however, recent studies also show an increased risk for development of therapy-related hematologic malignancies. Models of leukemogenesis propose that clonal hematopoiesis may be an early marker of genetic damage, preceding the acquisition of critical, recurring genetic alterations associated with the development of therapy related myelodysplastic syndromes and acute leukemia. The goal of this study is to determine whether dose-intensive adjuvant regimens for breast cancer induce genetic damage to hematopoietic stem cells, defined by the emergence of clonal hematopoiesis in a subset of patients following dose-intensive adjuvant therapy for brease cancer. Clonal hematopoiesis will be evaluated using two different methods, the HUMARA clonality assay (based on a methylated X-linked polymorphism) and microsatellite instability assays. In year one, the following objectives were completed: a) activation of the clonal hematopoiesis biological protocol (S9719), b) introductory protocol presentations at the SWOG Fall 1997 and Spring 1998 meetings, c) advertisements mailed to SWOG institutions including revisions of model consent form, d) beginning of patient registration, e) development of a centralized specimen collection/processing repository, f) standardization of the HUMARA and microsatellite assays, and g) addressing quality control and quality assurance issues.

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Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence

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SOUTHWEST ONCOLOGY GROUP
DAMD17-97-1-7088 ANNUAL REPORT

Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence

INTRODUCTION

Subject: Clonal Hematopoiesis As A Marker Of Genetic Damage Following Adjuvant Chemotherapy For Breast Cancer: Pilot Study To Evaluate Incidence. Southwest Oncology Group Study.

Study Purpose: The goal of this study is to **determine whether dose-intensive adjuvant regimens for breast cancer induce genetic damage to hematopoietic stem cells, defined by the emergence of clonal hematopoiesis.** This pilot study will test the hypothesis that genetic damage defined by the presence of clonal hematopoiesis can be detected in a subset of patients following dose-intensive adjuvant therapy on a current Southwest Oncology Group trial for breast cancer. To answer this question, we are studying sequential blood/bone marrow samples from 200 women enrolled in a single, randomized dose-intensive Southwest Oncology Group adjuvant breast cancer study for women with four to nine positive nodes (S9623, "A Comparison of Intensive Sequential Chemotherapy using Doxorubicin plus Paclitaxel plus Cyclophosphamide with High Dose Chemotherapy and Autologous Hematopoietic Progenitor Cell Support for Primary Breast Cancer in Women with 49 Involved Axillary Lymph Nodes, Phase III, Intergroup"). Two different assays (HUMARA and microsatellite instability) will be used to detect clonal hematopoiesis as a marker of genetic damage in this pilot study. **S9623** will compare the clinical outcome produced with autologous peripheral blood progenitor cell (PBPC) supported high-dose therapy with that of intensive, sequential chemotherapy in Stage II/III breast cancer patients with disease involving 4-9 axillary lymph nodes.

Scope of the Research:

1. To estimate the incidence of early genetic damage, defined by the presence of clonal hematopoiesis using a general clonality assay, the HUMARA (human androgen receptor assay), in pretreatment blood and bone marrow, apheresis, and two sequential post-treatment specimens in breast cancer patients enrolled in S9623.

2. To screen these samples for the presence of defective DNA mismatch repair mechanisms and the loss of heterozygosity, using assays to detect microsatellite instability as an alternative means of detecting a clonal population resulting from genetic damage following dose-intensive adjuvant regimens for breast cancer.

3. To estimate the incidence of MLL fusion gene transcripts in cases where either the HUMARA or microsatellite repeat assays are positive for clonal hematopoiesis, a finding commonly reported in t-AML with 11q23 abnormalities.

4. To determine the frequency of *RAS* gene mutations (*H-, K-,* and *N-RAS*) following dose-intensive adjuvant regimens for breast cancer.

Background: Adjuvant therapy with anthracycline-based combination chemotherapy for patients with breast cancer has been shown to improve disease-free and overall survival. Unfortunately, therapy-related myelodysplasia (t-MDS) or therapy-related acute myelogeneous leukemia (t-AML) has emerged as uncommon, but well-established, complications of adjuvant therapy using dose-intensive regimens for breast cancer. (1-3) t-MDS and t-AML evolve as a result of expansion of an abnormal clone of hematopoietic stem cells, which have acquired somatic mutations conferring a growth advantage.

According to the Jacobs model for leukemogenesis, mutations resulting in clonal hematopoiesis may occur without any obvious hematological change (no dysplasia or cytopenias noted). Subsequently, the acquisition of a variety of additional genetic lesions may be essential for the development of MDS (preleukemia) or overt leukemia. Clonal chromosomal abnormalities have been reported in the majority of cases of t-MDS/AML. The most frequently reported abnormalities involve complete loss or interstitial deletions of the long arm of chromosomes 5 and/or 7. Typically, these leukemias develop, following alkylating agent-induced damage, at a median of 3 - 5 years following therapy. (5) The second group of t-MDS/AML is associated with rearrangements of the MLL gene localized to chromosome band 11q23. (6-9) The 11q23-associated t-AMLs often develop following treatment with drugs that target DNA-topoisomerase II (e.g., epipodophyllotoxins and anthracyclines), with a very short latency (12 to 18 months) following treatment. (8)

Over the last ten years, anthracyclines have become a major component of combination chemotherapy regimens for breast cancer. Two recent adjuvant breast cancer trials, NSABP-B25 and NCIC, employing dose-intensive anthracycline-based chemotherapy, reported rates of t-MDS/AML that are two to four-fold higher than in previous adjuvant studies. These monocytic leukemias occurred following a short latency period (within two years of adjuvant therapy), a characteristic finding of hematologic disorders linked to DNA topoisomerase II inhibitors. Cytogenetic analysis revealed rearrangements of 11q23 in five of eight cases. Further concern about the development of t-MDS/AML following high-dose chemotherapy (with or without stem cell rescue) for breast cancer may be warranted, based on the alarming rates t-MDS/ AML following autologous transplantation for lymphoma, where the incidence of therapy-related leukemias has been estimated to be as high as 18% at six years following transplantation. (10-14)

These studies raise three major concerns: 1) Does genetic damage, leading to the development of clonal hematopoietic stem-cell disorders, occur with unacceptable frequency in patients receiving intensive anthracycline-based adjuvant regimens for the treatment of breast cancer? 2) Will careful monitoring of this patient population reveal additional cases of t-MDS/AML with long-term follow-up? and, 3) Does administration of recombinant hematopoietic growth factors (G-CSFs), used to minimize morbidity and facilitate scheduled drug dosing, play a potentiating role in the development of these secondary malignancies?

Chemotherapeutic agents used in the treatment of breast cancer may induce genetic damage. This damage may result in clonal proliferation that according to the Jacobs model of neoplasia, is an essential early, or possibly initial, step in leukemogenesis, occurring prior to the development of clinical abnormalities. Data confirming the presence of clonal proliferation following chemotherapy exist. Carter and others described clonal hematopoiesis in more than 30% of 70 clinically asymptomatic patients who had received prior cytotoxic chemotherapy for lymphoma. (4, 15, 16) Busque *et al.* found that clonal hematopoieses existed in 8 of 12 (66%) patients with Hodgkin's or Non-Hodgkin's lymphomas, studied prior to autologous transplantation (all had received prior chemotherapy), and that this value was significant ($p < 0.0033$) when compared to normal control donors. (17) Gale *et al.* have shown that sequential X-linked clonality assays are predictive of subsequent evolution to frank MDS/AML. (18) These provocative studies suggest that clonal hematopoiesis following chemotherapy may be a relatively common event. Pilot studies are warranted to determine the clinical relevance of these interesting findings.

The development of clonal hematopoiesis may be one of the earliest events that occur in an evolving neoplastic process. (18) Thus, assays to detect clonality, such as the PCR-based HUMARA (human androgen receptor assay), may define primary steps in the evolution to t-MDS/AML. (19, 20) Because the HUMARA assay is informative in more than 90% of females, it is probably the optimal clonality assay for testing female blood or marrow samples for clonal hematopoiesis at regular intervals. (21) Genomic instability at simple repeated DNA sequences, or microsatellites, is a sensitive marker of a genetic change resulting from genetic damage. (22-23) It appears that instability in these repeated sequences is

a result of defective DNA replication/repair mechanisms. In two recent publications, microsatellite variants arising from genomic instability were used as clonal markers in hematologic malignancies. (24-25) Therefore, the microsatellite instability and the HUMARA assays are complementary PCR-based methods of detecting genetic damage, and can be done using a very small amount of DNA obtained from blood. To determine the incidence of specific genetic lesions following high dose intensive adjuvant regimens for breast cancer, MLL gene rearrangements and *RAS* mutations, genetic alterations frequently observed in therapy-related hematopoietic disorders will also be evaluated. The HUMARA will serve as a general clonality assay, while the microsatellite instability assays will assess genetic damage at specific chromosomal loci in genomic regions associated with the development of tMDS/AML.

BODY

Experimental Design: This pilot study is designed to test the hypothesis that genetic damage defined by the presence of clonal hematopoiesis can be detected in a subset of patients following dose-intensive adjuvant therapy on a current Southwest Oncology Group trial for breast cancer. To answer this question, we have chosen to study sequential blood/bone marrow samples from 200 women enrolled on a single, randomized dose-intensive Southwest Oncology Group adjuvant breast cancer study for women with four to nine positive nodes (**S9623**, "A Comparison of Intensive Sequential Chemotherapy using Doxorubicin plus Paclitaxel plus Cyclophosphamide with High Dose Chemotherapy and Autologous Hematopoietic Progenitor Cell Support for Primary Breast Cancer in Women with 49 Involved Axillary Lymph Nodes, Phase III, Intergroup").

Statement Of Work Objectives/Problems: Upon notification of Department of Defense (DoD) funding, Drs. Slovak, Stock, Green and Willman, drafted the biological protocol entitled, "Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence" as a companion study to the active Southwest Oncology Group adjuvant treatment protocol (S9623), following Group protocol development and administration guidelines. The Southwest Oncology Group Operations Office and Statistical Center assisted in protocol development and devised a mechanism to offset institutional mailing costs for blood and bone marrow samples. To ensure the study was consistent in content and contained all the information required by the National Cancer Institute (NCI), the protocol was centrally reviewed, modified and approved by the Southwest Oncology Group Scientific Advisory Board and the Breast Cancer and Bone Marrow and Stem Cell Transplantation Committee Chairs, Dr. Silvana Martino and Dr. Stephen J. Forman, respectively. Subsequently, the study was submitted to the NCI (Cancer Therapy Evaluation Program or CTEP) and approved on August 22, 1997 (see Exhibit 1). DoD approval then followed.

The Southwest Oncology Group clonal hematopoiesis protocol (S9719, see Exhibit 2) was activated on October 15, 1997. Official notification to all Southwest Oncology Group member institutions occurred with the November 1, 1997 mailing. Introductory protocol presentations were made by Drs. Slovak and Stock to the Breast Cancer, Bone Marrow and Stem Cell Transplantation, and Leukemia Biology Committees at the Southwest Oncology Group Fall 1997 and Spring 1998 meetings. In addition, Drs. Slovak and Stock mailed advertisements to Southwest Oncology Group transplantation principal investigators to introduce the protocol's goal and significance (see Exhibit 3). Telephone calls with follow-up letters to institutional principal investigators are in progress to update Southwest Oncology Group clinical investigators of consent form revisions, protocol amendments, and specimen submission reimbursement information.

To assist with accrual goals, an invitation requesting intergroup participation to the Cancer and Leukemia Group B (CALGB) was sent to Drs. L. Norton and Daniel Hayes (see Exhibit 4). Dr. D. Gary Gilliland, Chair of the CALGB Leukemia Biology Committee has indicated CALGB may be interested in intergroup participation. Dr. Wendy Stock is currently working out the logistics with Dr. Gilliland.

Due to concerns from Southwest Oncology Group member institutions, steps to simplify participation in

the study have been taken. On 12/1/97, the DoD had mandated the addition of the following statement in the informed consent form (see Exhibit 5):

> **You are authorized all necessary medical care for physical injury or disease which is determined to be the proximate (or direct) result of your participation in this research study. The U.S. Army, which funds this study, requires that such medical care is provided by the local research institute when conducting research with private citizens. Other than medical care that is provided for physical injuries or disease determined to be a direct result of your participation on this trial, you will not receive any compensation for participating in this research study; however, this is not a release or waiver of your legal rights.**

Many Southwest Oncology Group institutions objected to this mandatory paragraph and refused to submit the clonal hematopoiesis protocol to their local institutional IRBs until a revision to delete this paragraph was made. This DoD mandate resulted in low patient accrual for year one. The Southwest Oncology Group contacted the DoD about this unfortunate situation and a revision to the model consent form was permitted. Notice of the revised consent form occurred on June 15, 1998. It is currently unknown how many Southwest Oncology Group institutions have local IRB approval. Five independent Southwest Oncology Group affiliates have registered patients to this protocol.

As a result of our preliminary findings (see below, Experimental Methods and Preliminary Results) and feedback from member institutions, an amendment to the protocol eliminated the requirement for buccal smear samples, made the pre-treatment bone marrow sample optional, and reduced the required amount of blood collected. Amendment 2 with a second protocol announcement was distributed to Southwest Oncology Group institutions on September 1, 1998 (see Exhibit 2).

The project is currently completing its first year of intensive activity. The Statement of Work underestimated the amount of time needed for the protocol development and approval process. There are numerous levels of approval required by both the Southwest Oncology Group, the NCI and the DoD, with subsequent lag times between deadlines for submission and distribution of materials, review, receipt of feedback, and resubmission. The Southwest Oncology Group and NCI gave this protocol priority status, personnel made every effort to facilitate the approval process, and the protocol was favorably received at every point at which it was received. As discussed above, the DoD informed consent mandate issued in December 1997 resulted in objection by Southwest Oncology Group member institutions. This latter concern was resolved in June 1998 but unfortunately, resulted in a six-month delay in patient accrual. It is clear that this process takes longer than estimated, even when given priority handling. For these reasons, we would like to raise the possibility of extending patient registration for an additional six to nine month period to ensure receipt of the last sample (12 months after the completion of all therapy). The study has received positive feedback whenever it has been presented, and both clinicians and patients are strongly enthusiastic about the potential of this project to contribute to breast cancer. For these reasons, we are confident that the project will be completed as proposed, even though initial activities took longer than planned.

Experimental Methods and Preliminary Results

Sample collection: Despite the consent form concerns listed above, five patients were registered to the clonal hematopoiesis protocol in year one. IRB approval was obtained at the following Southwest Oncology Group institutions: Salem Hospital (164192), St Francis/Stormont (164513), Henry Ford Hospital (164598), N Colorado Medical Center (163093), and Oregon Health Sciences University (163674). Blood and bone marrow paired pretreatment samples were obtained from all five patients.

DNA samples from each of the 200 patients enrolled will be studied at the timepoints as outlined in Table 1. Two ml of a pretreatment bone marrow aspirate from each patient (required for entry to S9623) is

requested, with collection into EDTA tubes. This sample serves as a sensitive control for the detection of any pre-treatment abnormality. Forty ml of blood will be collected from registered patients prior to initiation of treatment, and at three and twelve months following completion of all chemotherapy. For the 100 women in our study who are randomized to the autologous stem cell transplant arm of S9623, 2 ml from the stem cell collection will also be obtained for analysis. All samples are sent at room temperature by overnight courier to arrive within 24 hours at the Southwest Oncology Group tissue repository at the University of New Mexico, directed by Cheryl L. Willman, M.D., where samples are processed for DNA and RNA. Additional cells are cryopreserved. Samples are subsequently batched and sent to the University of Illinois at Chicago for microsatellite instability and *MLL* RT-PCR assays (Dr. Wendy Stock) and the City of Hope National Medical Center for the HUMARA assay (Dr. Marilyn Slovak). *RAS* mutation studies will not be performed until year three. The repository at the University of New Mexico retains frozen cells and DNA on all samples for future studies.

TABLE 1. Time points for Sample Collection for Study of Clonal Hematopoiesis (Arm A – intensive chemotherapy; Arm B – autotransplant)

Time points		Time points	Sample Source
A.	Pretreatment	Arms A + B (200 Women)	Bone Marrow Blood
B.	Stem Cell Collection	Arm B only (100 Women)	Apheresis (peripheral blood stem cells) or Bone Marrow Blood
C.	3 Months Following Completion of All Chemotherapy	Arms A + B (200 Women)	Blood
D.	12 Months Following Completion of All Chemotherapy	Arms A + B (200 Women)	Blood
E.	In case of diagnosis of Secondary Malignancy	Arms A + B	Bone Marrow Blood

*If the patient is also registered to S9702, pretreatment and stem cell collection samples may be collected for that study at the same time.

Sample Processing: High molecular weight DNA is prepared from the blood and apheresis samples following Ficollgradient separation, following standard proteinase K digestion and phenol/chloroform extraction methods. (26) Cells from each blood sample (a minimum of 20-30 x 10^6 cells) will be frozen for viability according to standard methods. (27)

HUMARA assay: The HUMARA assay is a PCR-based test for detecting clonality utilizing the human androgen receptor locus on the X chromosome. The assay takes advantage of dosage compensation in humans achieved through random inactivation of one of the two X-chromosomes in the cells of normal females. This inactivation occurs early in development and thus females are essentially cellular mosaics for the genes on the X chromosome, that is, some cells have either the maternal or paternal X chromosome inactivated. This genetic concept is also known as the Lyon hypothesis. In theory, all normal female tissue would be randomly methylated with 50% of cells having the paternal (pat) allele inactivated and 50% of the cells having the maternal (mat) allele inactivated; the ratio of relative methylation would be 50:50, or a ratio of 1. This pattern of inactivation is maintained faithfully in all progeny. (20)

The second important feature of the androgen receptor locus is the multiallelic CAG trinucleotide repeat in the first exon of the human androgen-receptor gene. This CAG repeat is polymorphic in 90% of females of all racial groups. (28, 29) Consequently, the informativeness of this assay, due to 20 different alleles, coupled with the ability to quantitate allelic ratios between the active and inactive X chromosome, makes the HUMARA assay an optimal clonality assay to assess clonal hematopoiesis at regular intervals.

The HUMARA assay has been validated by several investigators and found to be reliable and reproducible in studies of human neoplasia. (18, 30-35) In a retrospective analysis of 104 female patients from a single institution undergoing autologous bone marrow transplantation (ABMT) for NHL, polyclonal hematopoiesis was identified in 77%, a skewed chromosome X-inactivation pattern was observed in 20% and 3% or 3 patients had clonal hematopoiesis at the time of BMT. (35) To further test the predictive value of clonality for the development of t-MDS/AML, a subgroup of 78 patients with at least 18 months of follow-up, was evaluated. In this subgroup, 53 patients remained polyclonal, 15 had skewed X-inactivation, and 10 had clonal hematopoiesis at time of ABMT or developed it after ABMT. The results of the retrospective study were that a significant number of patients have clonal hematopoiesis at ABMT. In fact, clonal hematopoiesis predicted the development of t-MDS/AML in 3 of 4 patients seven to 15 months before clinical diagnosis of disease. (35)

Clonality at the HUMARA locus is assessed by nested PCR amplification, according to Mach-Pascual *et al.* and quantitated by the method of Delabesse *et al.* (35, 36) The HUMARA assay is designed to amplify a ~250 to 300 base pair (bp) region of the first exon of the human androgen receptor. Two *Hpa II* methylation sensitive sites reside within 100 bp 5' to the polymorphic CAG repeat. Primers flank the methylation sensitive restriction enzyme sites and the CAG repeat simultaneously. Methylated enzyme (*Hpa II*) sites correlate with X inactivation. Unmethylated alleles (active X) are digested by *Hpa II* and eliminated from PCR amplification. The methylated or inactive allele will remain intact after the *Hpa II* digestion and is the only allele amplified. After amplification, the maternal and paternal alleles are resolved using a sequencing gel. Random inactivation will show both maternal and paternal alleles, signifying a polyclonal state; whereas a clonal population will be identified by the presence of one allele or a shift of greater than 3-fold, to control for skewed X-inactivation over the other allele.

To address quantitative aspects, interlaboratory variability, quality control and reproducibility issues, a collaboration was established with D. Gary Gilliland, M.D., Ph.D., Brigham and Women's Hospital, Harvard Institutes of Medicine, Harvard Medical School, Boston, MA. Dr. Gilliland provided two DNA panels to Dr. Slovak's laboratory to run in a blinded fashion. The first panel was composed of a mixture of 12 polyclonal or clonal DNA samples with differences between mat and pat alleles, ranging between 3 to 40 bp. The 5' primer was labeled with fluorscein, with quantitation of alleles performed using a Fluor-imager and ImageQuant software. This study was performed in a blinded fashion to obtain the highest degree of confidence and best design for the pilot study. Samples were run in duplicate and the entire assay was repeated twice to ensure reproducibility. Figure 1 shows the results of seven DNA samples. Each sample comprises four lanes; the first two lanes contain DNA digested with *Rsa I* and lanes 3 and 4 were digested with both *Rsa I* and *Hpa II*. *Rsa I*, a restriction enzyme that is not sensitive to DNA methylation status, cuts the DNA outside the trinucleotide repeat of the human androgen receptor gene locus, allowing for visualization of both alleles based on their differences in CAG repeat number. Lanes 1 and 2 of each sample are digested with *Rsa I* only and show both mat and pat alleles. Lanes 3 and 4 have been digested with both *Rsa I* and *Hpa II*, which determines inactive allele status. In Figure 1, samples 2381, 2383, 2413 and 2422 exhibit polyclonality, whereas clonal hematopoiesis has occurred in samples 2389, 2395 and 2404.

Figure 1

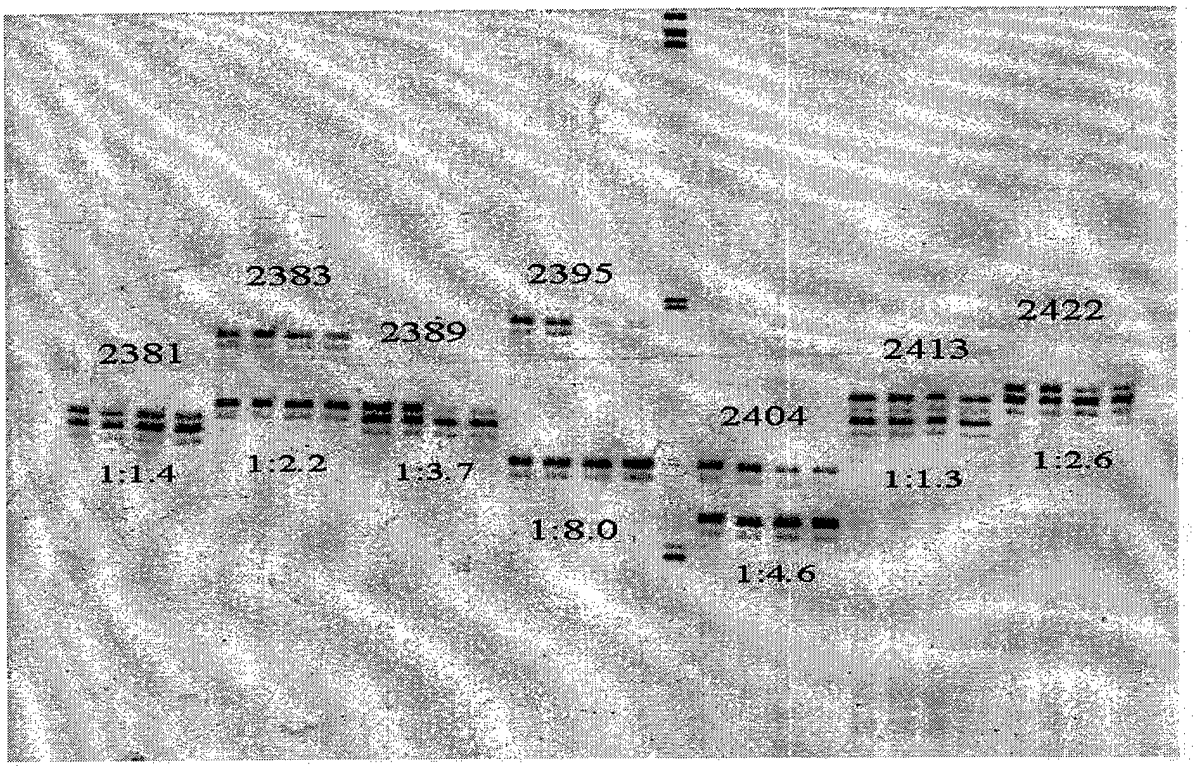

Figure 1. This gel represents a random sampling of female patients who received autologous bone marrow transplants post chemotherapy for non-Hodgkin's Lymphoma. Samples 2381, 2383, 2413 and 2422 show polyclonal hematopoiesis. Samples 2389, 2395 and 2404 show clonal hematopoiesis with allelic ratios ≥ 3:1.

The allele ratio is defined as the ratio between the two chromosome X-linked alleles in a given sample. The corrected ratio is determined by dividing *Rsa* I-*Hpa* II by *Rsa* I alone. This corrects for preferential amplification of one of the two alleles. Those samples with a corrected ratio of less than 3 are considered within normal limits. Those with a corrected ratio greater than 3 are consistent with either skewed X inactivation or clonal hematopoiesis. Although criteria for nonrandom X-inactivation are arbitrary, an allele ratio ≥ 3:1, which corresponds to the expression of 75% of one allele, has been widely accepted in the literature. (20, 34, 35, 37-39) To normalize for excessive skewing, which occurs in ~20% of the female population and appears to increase with aging, the ratio of the two alleles in the experimental tissue (polymorphonuclear cells) must be divided by the ratio of the same two alleles in normal somatic control tissue (T-cells). (37) If the ratio remains greater than 3, the results are consistent with clonal hematopoiesis.

Ten of twelve sample ratios in the blinded study were in agreement. In order to identify the reason(s) for

the two discrepancies, Ms. Victoria Bedell, Research Associate at the City of Hope National Medical Center, visited Dr. Gilliland's laboratory in Boston. The studies were repeated in the Dana-Farber Laboratory. Dr. Gilliland's laboratory used a radioactive detection method; Dr. Slovak's laboratory used a fluorescent detection method. The two discordant samples were run a third time. The allelic ratios from the fluorescent technique were found to be more accurate, allowing better resolution of fragments with only a 3-6 base pair size difference. Today, both laboratories use a standardized, fluorescent HUMARA assay.

In our preliminary studies, two problems were identified that will require careful control. Low sensitivity is the first limitation of this general clonality assay. Willman *et al.* have performed mixing experiments which demonstrate that the percentage of clonal cells can be estimated with an error of ± 10%, and a clonal population of cells can be detected if they constitute more than 10% of the cells in a polyclonal background. (21) Thus, for the HUMARA assay to recognize a clonal population, the population must represent more than 10% of the total experimental tissue cells above normal background. Regardless, a recent study from Dana Farber has shown that the presence of clonal hematopoiesis is predictive for development of t-MDS in non-Hodgkin's patients who have been treated by various dose intensive regimens, including autologous bone marrow transplantation. (35) Despite the issue of low sensitivity, the HUMARA assay is a highly specific general clonality assay to identify clonal hematopoiesis post high dose chemotherapy.

The second limitation of the HUMARA assay that requires proper control is excessive lyonization. This refers to a skewed X-inactivation that occurs in females who have randomly inactivated a preponderance of one X-chromosome (either paternal or maternal X). Gale and colleagues have estimated this occurs in ~23% of females and has been reported to increase with age, with >30% of the normal population having skewed XIP at age 60. (37, 38) Because X inactivation patterns may vary from tissue to tissue, somatic controls from embryologically related tissues are needed to determine and interpret skewed X inactivation patterns. (38) Initially, we began our study using DNA extracted from buccal mucosa preparations, however, a false positive was identified. Subsequently, we determined that T cells as the most promising control for skewed X-inactivation. Recently, others have reported similar results. (29) The use of T cells, as the control tissue will help eliminate false positives due to skewed X-inactivation that mimic a clonal population.

Accordingly, we have optimized a procedure for isolating T-cells and polymorphonuclear cells from peripheral blood samples. Through the use of immunomagnetic beads, we are able to isolate T cell DNA. Granulocyte contamination is less than 1%, which has been verified by flow cytometry. (DYNAL) Four 7 ml tubes (28 ml) of sodium heparinized blood are necessary to obtain an adequate amount of T-cells for use as a control. PCR products were obtained for the four T-cell controls and polymorphonuclear cells isolated in our lab. In addition to optimizing T-cell isolation, we needed to determine the best method for storing and transporting the samples. Using identical samples, one specimen was stored overnight at 4° in 20 ml of RPMI 1640 tissue culture medium. The second sample, also supplemented with 20 ml RPMI medium, was stored overnight at room temperature. Both yield and purity of the DNA was better in the sample stored at 4°.

Figure 2

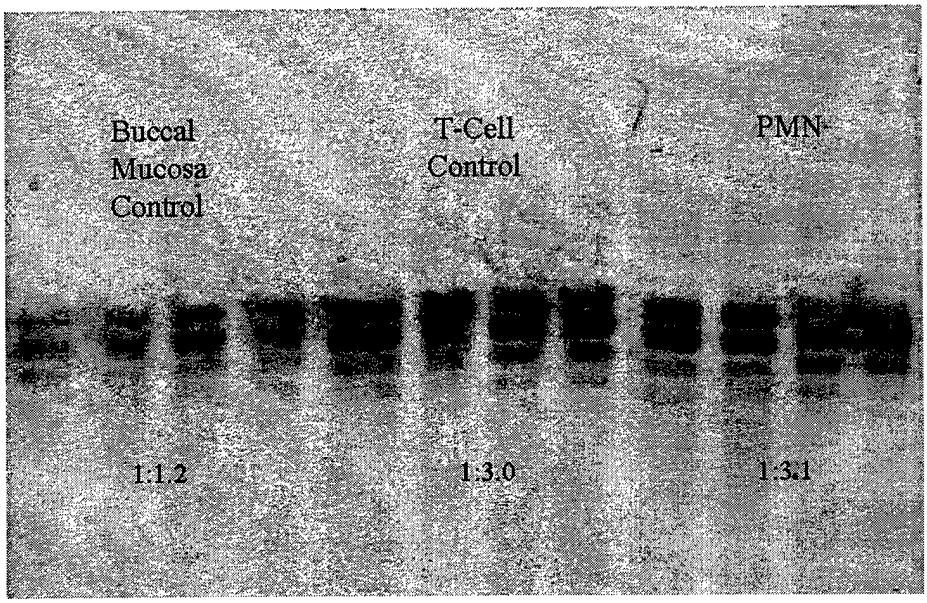

Figure 2. Comparison of control tissue for the HUMARA assay. This gel demonstrates the necessity for control and experimental DNA to be derived from the same embryonic tissue. The allelic ratio of DNA from buccal mucosa tissue indicates a polyclonal population, however analysis of T cell DNA indicates that constitutional excessive lyonization has occurred in this individual's hematopoietic cells. Polymorphonuclear (PMN) cells exhibit a similar allelic ratio to the T cell DNA, confirming that different tissues in an individual may have different X- inactivation patterns.

Both 4% and 6% acrylamide gels were run on identical PCR samples to determine an optimal acrylamide concentration. Although 4% gels have better resolution in gels run for three hours, 6% gels have equal resolution, as well as a crisper-banding pattern, when run for four hours. The acrylamide concentration did not change the ratios of the samples.

Per our request, Dr. Gilliland provided a second panel of sequential DNA samples, over a three-year period from a patient with evolving clonal hematopoiesis. This blinded study ensures our capability of identifying shifts in allelic ratios over time in patient follow up samples. Figure 3 shows the results of a set of sequential samples from patient #2181, diagnosed with lymphoma and receiving an ABMT before 1995 at Dana Faber. Samples C, I and J were annual samples collected from this woman in years 1993, 1994 and 1995 respectively. Please note the 1993 sample designated as "C," is polyclonal with a corrected ratio of 1.2. In years 1994 and 1995, the shift in intensity of the bottom alleles indicates the presence of clonal hematopoiesis. Once a patient exhibits clonality, she remains clonal consistently, and over time, there is an increase in the clonal population presumably due to selective growth advantage.

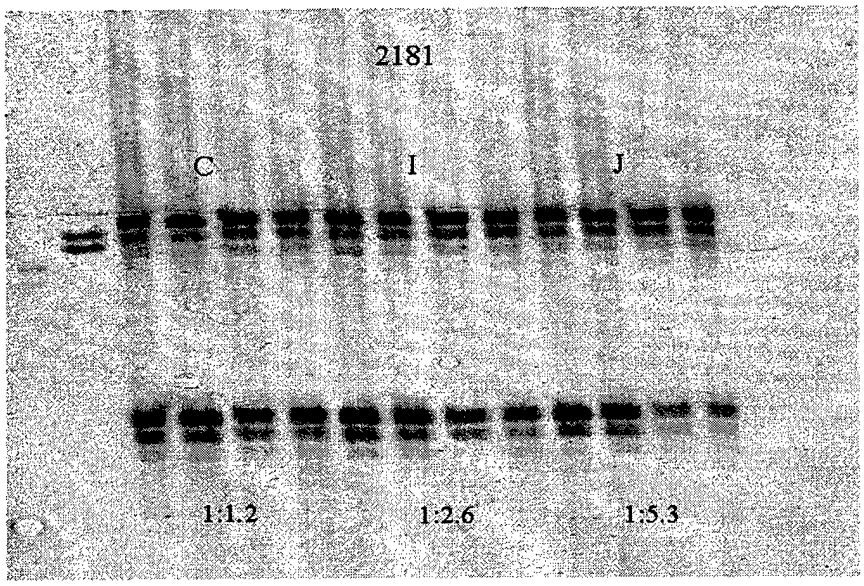

Figure 3. Sequential samples from patient #2181. This gel illustrates three annual samples from a NHL patient who underwent transplantation in 1993. Sample C (lanes 2-5) was isolated post transplant in 1993. The patient demonstrates polyclonal hematopoiesis with a ratio of 1:1.2. In 1994 the patient has a slight shift in the allelic ratio 1:2.6 (lanes 6-9), however, the shift is not large enough to indicate a true clonal population. In 1995, clonal hematopoiesis has occurred, with a significant shift of 1:5.3 (lanes 10-13). Lane 1, DNA markers, lanes 2, 3, 6, 7, 10, 11 are RSA I digestion only, lanes 4, 5, 8, 9, 12, 13 are digested with *Rsa* I and *Hpa* II.

In year one, all quality control and quality assurance issues for the HUMARA assay have been resolved.

Microsatellite Instability Assays: Microsatellite instability (MSI) assays have been chosen as an alternative method to the HUMARA for detection of a clonal hematopoietic stem cell population emerging as a result of chemotherapy-induced genetic damage. Since receiving funding from the DoD, we have identified 10 MSI markers and have developed conditions for amplification and detection of these markers in tumor (granulocytic fraction of blood or mononuclear fraction of bone marrow) and control (buccal mucosa or peripheral blood T cell) populations. (40-44) The first 5 MSI markers were chosen on the basis of existing literature, documenting their utility in the detection of MSI in a variety of different malignancies. These markers include BAT26, BAT40, APC, Mfd15CA, and D2S123. The other five markers are also highly informative and were chosen on the basis of their location in genomic regions where chromosome translocations or loss of heterozygosity (LOH) have been frequently reported in therapy-related leukemias and, specifically, where abnormalities have been associated with topoisomerase II inhibitors. These MSI markers (with genomic location) include AFM240YA11 (3q21), AFM302xb9 (11q23), AFM031xc5 (21q22), AFM337zg5 (12p12) and AFMb298yh5 (20pter-20qter).

During the past year, we have standardized PCR conditions for the first five markers and are now working on the second group of MSI markers. The specific conditions for each MSI marker are outlined below:

Microsatellite Markers:

BAT40

5'-ATT AAC TTC CTA CAC CAC AAC-3'
5'-GTA GAG CAA GAC CAC CTT G-3'

Location: 1p13.1

PCR conditions: annealing temp.: 58°C
$MgCl_2$ concentration: 1.5mM
Primer amount/reaction: 5pmol
Template amount/rxn: 100ng

Amplicon size: ~80-100bp

BAT26

5'-TGA CTA CTT TTG ACT TCA GCC-3'
5'-AAC CAT TCA ACA TTT TTA ACC C-3'

Location: 2p

PCR conditions: annealing temp.: 55°C
$MgCl_2$ concentration: 2.5mM
Primer amount/reaction: 5pmol
Template amount/rxn: 100ng

Amplicon size: ~80-100bp

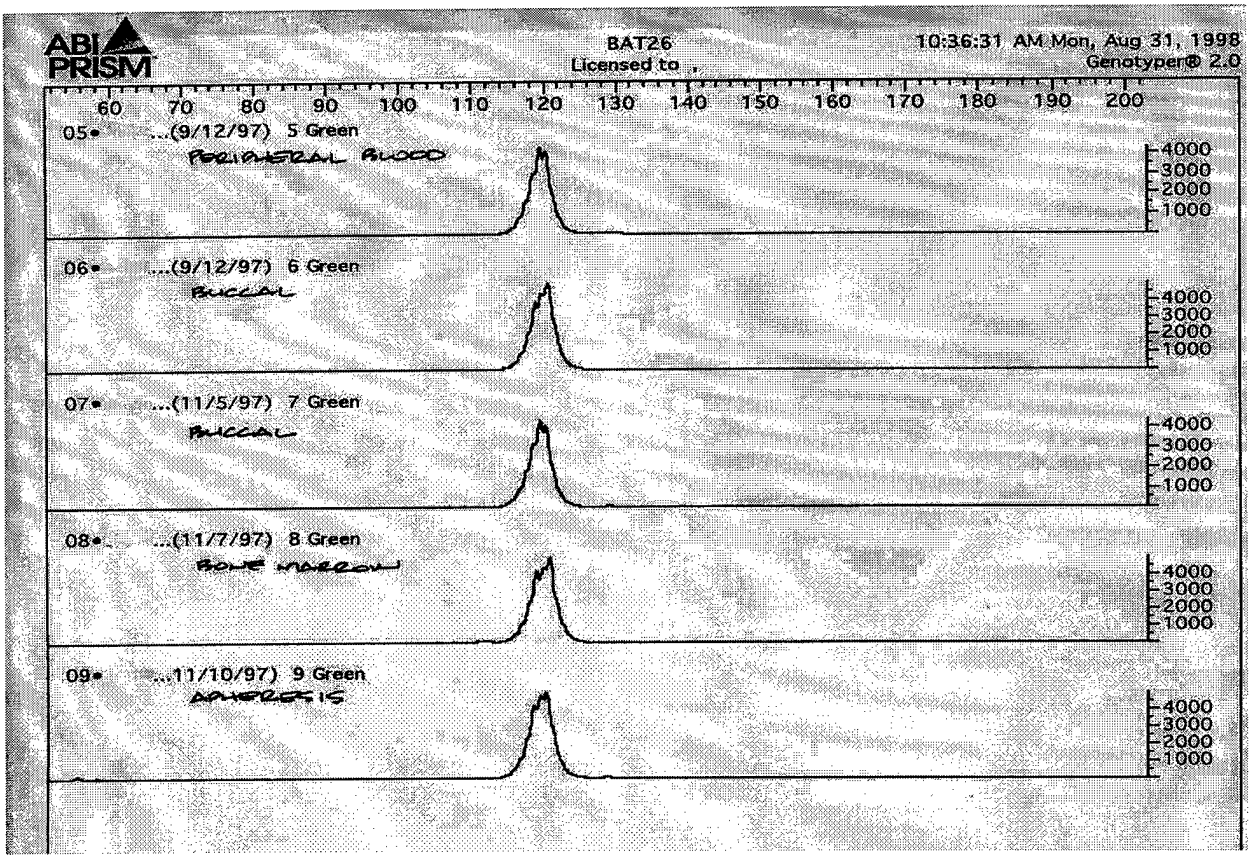

Figure 4. Microsatellite instability profile for BAT 26. All five samples submitted and assayed from this breast cancer patient are negative for instability at the BAT 26 locus.

AFM302xb9
(D11S1345)

5'-TGC CAC AGT AAT ACA TGT GTG TAA T-3'
5'-TAG TCA GTG CTG AGC CCA TA-3'

Location: 11q23.3-24.2

PCR conditions: annealing temp.: 55°C
$MgCl_2$ concentration:
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon length: 235bp

APC

5'-ACT CAC TCT AGT GAT AAA TCG-3'
5'-AGC AGA TAA GAC AGT ATT ACT AGT T-3'

Location: 5q21/22

PCR conditions: annealing temp.: 58°C
$MgCl_2$ concentration: 3.5mM
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon size: 96-122bp

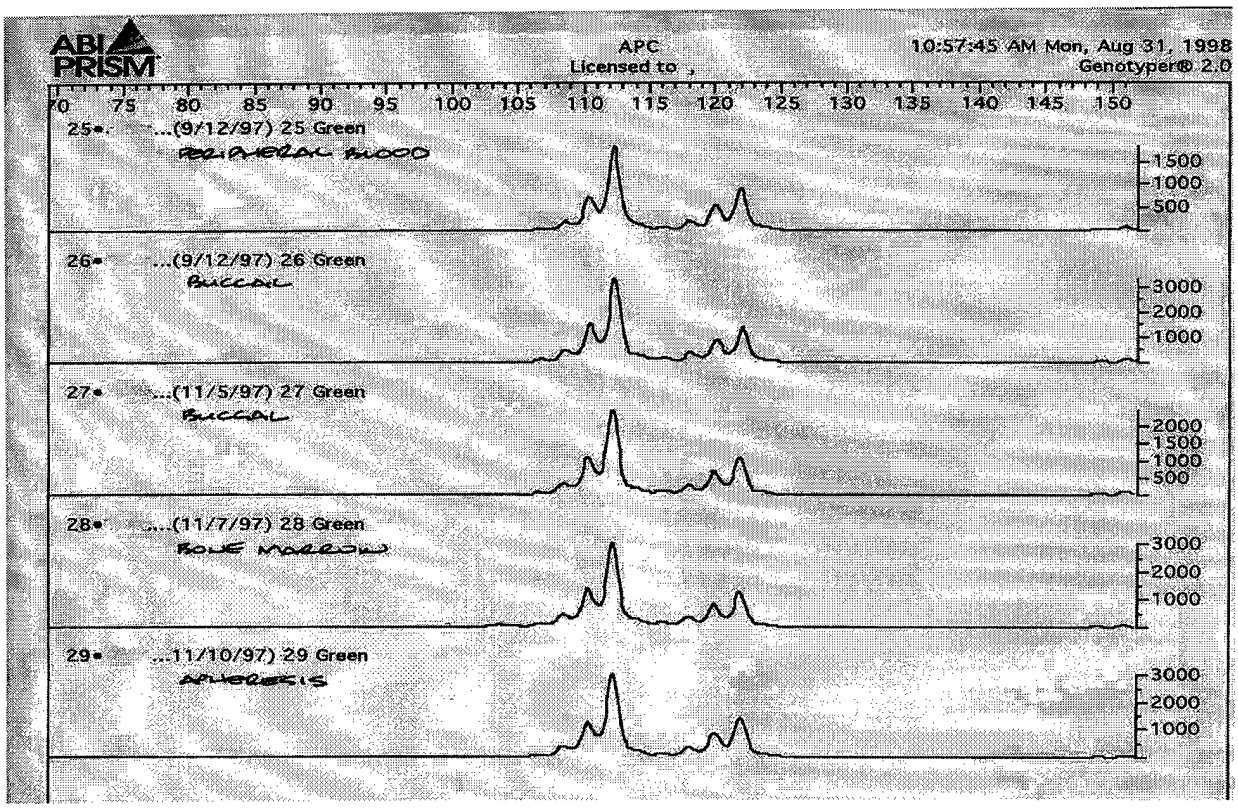

Figure 5. Microsatellite instability profile for APC from a breast cancer patient. All samples submitted to date fail to indicate instability at the APC locus.

AFM031xc5
(D21S270)

5'-GAA ATG TTT TAA TAA ATG GTG GTT A-3'
5'-ACA AAG TTA TGG TCA AGG GG-3'

Location: 21q22.2-21qter

PCR conditions: annealing temp: 55°C
$MgCl_2$ concentration:
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon length: 199-223bp

Mfd15CA

5'-GGA AGA ATC AAA TAG ACA AT-3'
5'-GCT GGC CAT ATA TAT ATT TAA ACC-3'

Location: 17q11.2-q12

PCR conditions: annealing temp.: 52°C
$MgCl_2$ concentration: 2.5mM
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon size: ~150bp

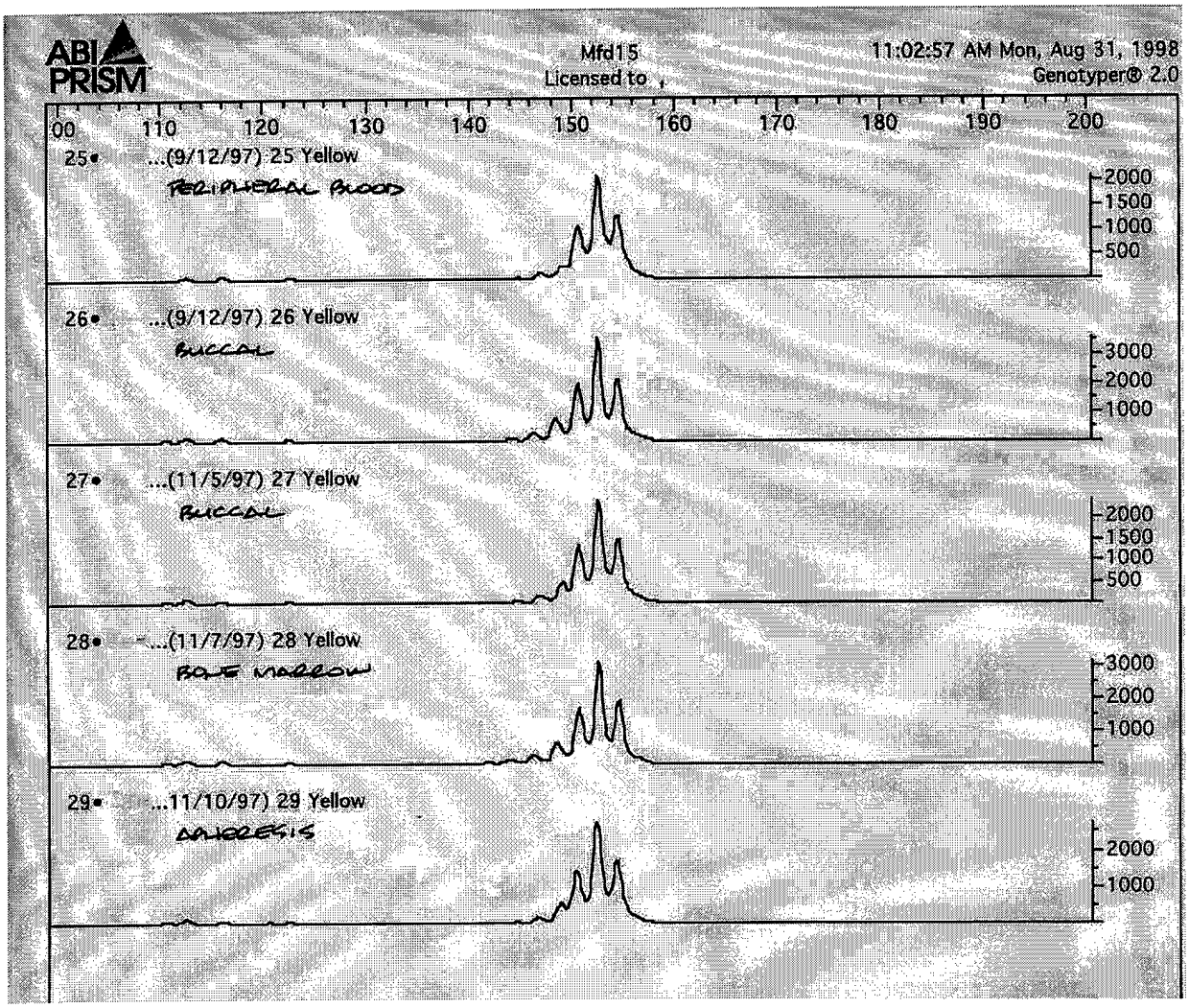

Figure 6. Microsatellite instability profile for mfd15CA from a breast cancer patient. The five samples submitted from this patient indicate no instability at the mfd15CA locus

AFM093xh3 5'-AAA CAG GAT GCC TGC CTT TA-3'
(D2S123) 5'-GGA CTT TCC ACC TAT GGG AC-3'

Location: 2p16

PCR conditions: annealing temp.: 60°C
$MgCl_2$ concentration: 2.5mM
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon length: 197-227bp

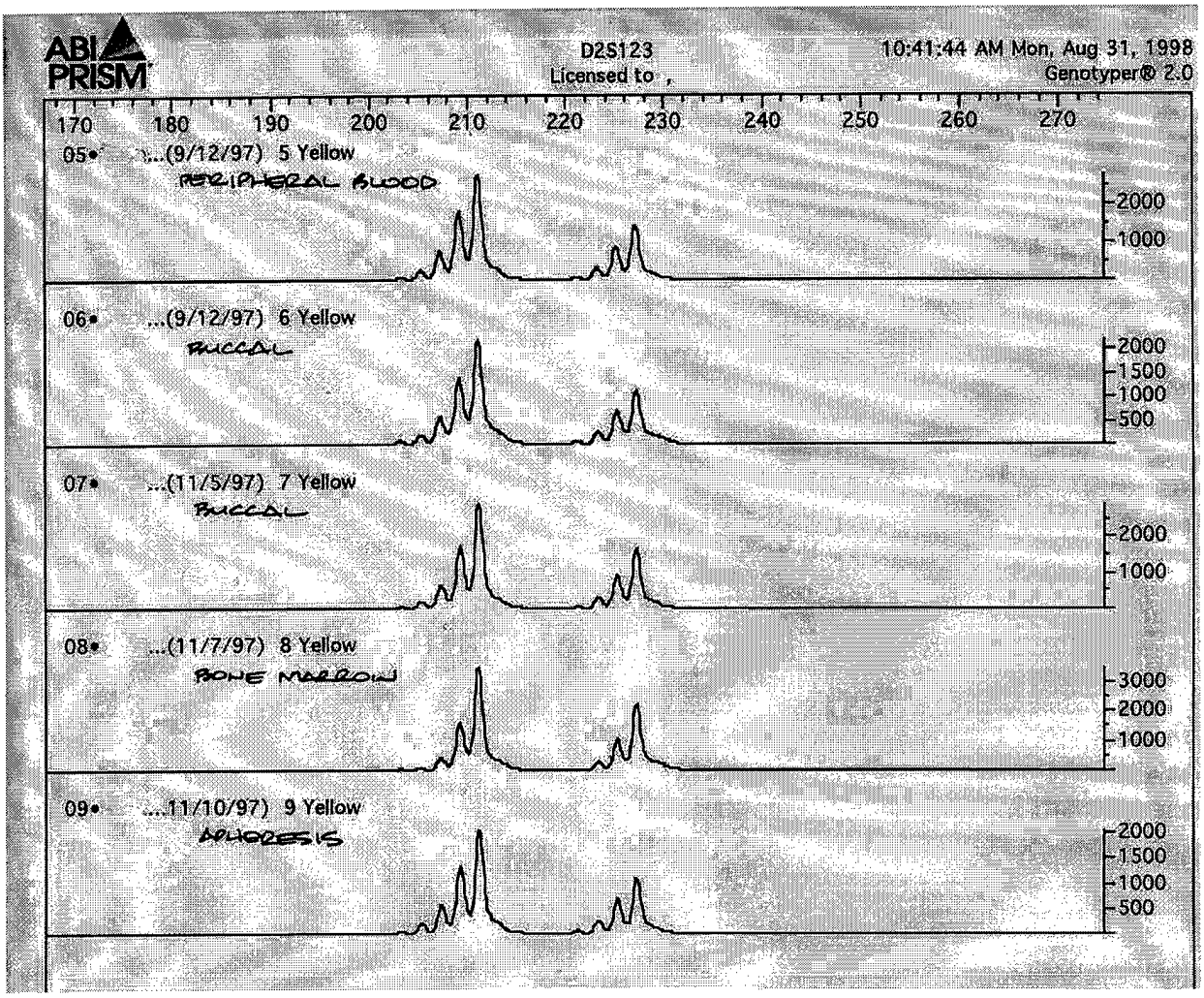

Figure 7. Microsatellite instability profile for D2S123. All samples submitted from this breast cancer patient are negative for instability at the D2S123 locus

AFM240YA11
(D3S1309)

5'-CTT TGG GGA ATC ATT AGT CTG T-3'
5'-ATG AGA ATT GTC ATG GTG C-3'

Location: 3q21-q25.2

PCR conditions: annealing temp.: 56°C
$MgCl_2$ concentration: 1.5mM
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon length: 141bp

AFM337zg5 (D12S363)

5'-GAG GGG TGG CAT CTC T-3'
5'-CTC AAA TGA AAT CAG CAT AAA-3'

Location: 12p12-p11.2

PCR conditions: annealing temp.: 55°C
$MgCl_2$ concentration:
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon length: 208bp

AFMb298yh5 (D20S887)

5'-TCA AGA GAT TTA TTG TCC AAC AAG-3'
5'-ATT GAG TGG GTT CAG ACT CC-3'

Location: 20pter-qter

PCR conditions: annealing temp.: 55°C
$MgCl_2$ concentration:
Primer amount/rxn: 5pmol
Template amount/rxn: 100ng

Amplicon length: 245-267bp

PCR primer pairs:

The forward primer of each primer set contains a 5'-end labeled fluorophore, which allow automated fluorescence detection. One fluorophore (6Fam, Hex, or Tet: blue, yellow, green fluorescence respectively) is end labeled to each forward primer.

PCR reactions:

All PCR reactions contain 100 ng of either normal (buccal) or tumor DNA and 2.5 units of Amplitaq Gold Polymerase (Perkin-Elmer). The PCR reaction is a "hot start" reaction; the Amplitaq Gold Polymerase must be heated initially at 95°C for 12 minutes in order to be activated for amplification.

Parameters: 95°C x 12 min

95°C x 30 sec
____ x 30 sec } 45 cycles
72°C x 30 sec

72°C x 10 min

25°C hold

Analysis:

2 µl of each PCR product is run on a 4% polyacrylamide denaturing gel:

Each product is combined with a 350 bp size standard (Perkin-Elmer) labeled with Tamra (red fluorescent fluorophore).

Gels are run on an ABI 377 instrument and are analyzed following electrophoresis using Genescan Analysis software. The Genescan software collects raw signals emitted by each fluorophore. Every fragment in a peak contributes a single fluorophore; peak area is directly proportional to the number of molecules. The Genotyper DNA Fragment Analysis Software aids in determining allele sizes of the amplified products. Therefore, control and "tumor" allele sizes can be compared for determination of microsatellite instability.

Detection of *RAS* mutations and *MLL* gene rearrangements: In cases where the HUMARA or microsatellite repeat assays are positive for clonal hematopoiesis, sensitive reverse-transcriptase PCR assays, using RNA from banked specimens, will be used to determine whether *RAS* mutations and/or *MLL* fusion transcripts commonly reported in therapy-related myelodysplasia and AML have occurred. (45-46)

Statistical Considerations: One hundred patients per arm from S9623 will be accrued on this study. The length of accrual is anticipated to be 2-3 years. For this pilot, samples will be obtained prior to initiation of treatment, from collected stem cell specimens (in 100 patients randomized to autologous stem cell transplant), and at three and twelve months following completion of treatment. T-cells will be collected at each timepoint and serve as an internal control for each patient sample. Currently, Dr. Stephanie Green and the Southwest Oncology Group Statistical Center are providing ongoing statistical study management and data management support.

Compliance with the three-month blood draw is expected to be nearly complete; at 12 months following completion of treatment, approximately 15% are anticipated to have relapsed or refused and not have samples available. The probability of clonal hematopoiesis at a particular timepoint can be established within ± 0.1 with a sample size of 100 per arm, and to within ± 0.11 with a sample size of 85. A two-sided .05 level test of the association of the treatment group with presence or absence of clonality will have adequate power to detect differences of .25 or greater (power at least .93 for the pretreatment and three month timepoint and .88 for the 12 month post-treatment timepoint, if sample size decreases to 85 per arm).

In the future, changes in status between pretreatment, stem cell collection and the three and twelve-month post-treatment samples will be explored, as will concordance of the HUMARA and microsatellite assays. Association of other pre-study patient characteristics and tumor-related variables, with presence or absence of clonality by HUMARA or microsatellite assays will also be explored.

CONCLUSIONS AND FUTURE DIRECTIONS

This pilot study was designed to test the hypothesis that genetic damage, defined by the presence of clonal hematopoiesis, can be detected in a subset of patients following dose-intensive adjuvant therapy on a current Southwest Oncology Group trial for breast cancer. The salient points outlined in the grant application's "Statement of Work" for year one have been completed on schedule. These objectives included: a) drafting and activating the clonal hematopoiesis biological protocol (S9719) entitled "Clonal Hematopoiesis As A Marker Of Genetic Damage Following Adjuvant Chemotherapy For Breast Cancer: A Pilot Study To Evaluate Incidence", as a companion study to the active S9623 adjuvant treatment

protocol, b) introductory protocol presentations held at the Southwest Oncology Group Fall 1997 and Spring 1998 meetings, c) mailed advertisements to Southwest Oncology Group institutions, revision of the model consent form per agreeable DoD and Southwest Oncology Group institution guidelines, d) beginning registration of patients to the protocol, despite the consent form quagmire, e) development of a centralized specimen collection and processing repository in the Southwest Oncology Group myeloid leukemia repository in New Mexico, f) standardization of the HUMARA assay and all quality control and quality assurance issues associated with this PCR-based assay addressed by performing a blinded analysis in collaboration with Dr. D. Gary Gilliland, and g) standardization of PCR conditions for the first five microsatellite markers to be used. To address the issue of low patient accrual, Drs. Slovak and Stock will call each Southwest Oncology Group institution with S9623 opened, to encourage registration to this very important biological protocol. In addition, Dr. Slovak will make follow-up presentations at the Southwest Oncology Group Fall 1998 meeting scheduled for October. Dr. Stock and Dr. Gilliland will determine the feasibility of having CALGB join the Southwest Oncology Group in this scientific effort to obtain additional patient samples to meet the statistical power requirements of this clonal hematopoiesis pilot study.

The confirmation that adjuvant chemotherapy induces clonal hematopoiesis in a significant number of patients from this pilot study will provide a unique model to prospectively study the evolution of therapy-related leukemogenesis in patients being treated for breast cancer, and would be the focus of a subsequent grant proposal. The goals of a larger study would include the following: 1) to determine whether a relationship exists between detection of clonal hematopoiesis and subsequent evolution to tMDS/AML; 2) to identify general mechanisms (e.g., faulty DNA repair and mutations in components of cell cycle checkpoints, which may predispose patients to genetic instability and leukemogenesis, following adjuvant therapy; 3) to determine the sequence of events (genomic instability, loss of heterozygosity, specific mutations/translocations, etc.) which participate in leukemogenesis; and 4) to determine whether specific adjuvant regimens place patients at an unacceptably high risk for the development of therapy-related hematologic malignancies.

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APPENDIX

Attached are the following appendices.

Exhibit 1: CTEP protocol approval letter

Exhibit 2: Clonal hematopoiesis (S9719) protocol with 09/01/98 revisions

Exhibit 3: Example of advertisement letter sent to Southwest Oncology Group institutions

Exhibit 4: Revision 1 describing the Department of Defense mandate to change the S9719 informed consent form (revision subsequently deleted on 06/15/98)

Exhibit 5: Invitation to ECOG and CALGB to participate in S9719

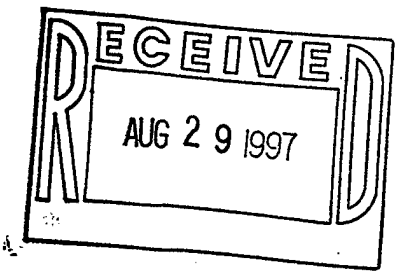
DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

NOTICE OF PROTOCOL APPROVAL with recommendations

Date: AUG 2 2 1997
(Revised Date: _____ PMB: ___)
NCI Protocol #: S9719
Local Protocol #: S9719
Protocol Chairman: SLOVAK,M.
Investigator #: 26404



Charles Coltman, M.D.
Group Chairman, SWOG
14980 Omicron Drive
San Antonio, TX 78245-3217

Dear DR. COLTMAN:

SWOG's protocol, "CLONAL HEMATOPOIESIS AS A MARKER OF GENETIC DAMAGE FOLLOWING ADJUVANT CHEMOTHERAPY FOR BREAST CANCER: PILOT STUDY TO EVALUATE INCIDENCE," has been approved and officially filed by the Cancer Therapy Evaluation Program, DCTDC, NCI.

As the Principal Investigator, it is your responsibility to inform all co-investigators and personnel involved in the study (data management, pharmacy, nursing, statistics, etc.) of the "approved" status of this protocol.

Enclosed are some recommendations for your consideration. Although you are not obligated to make changes in the present protocol document, you may wish to consider whether implementation of some or all of these suggestions would improve the study. We have submitted your current document to the FDA and/or PDQ. Should you elect to make any changes to the current document when you activate the study, please send an "Activation Amendment" (a list of each change to the approved protocol) to the Protocol and Information Office.

Notice of protocol activation (via the enclosed Protocol Status Update Form or an activation amendment) must be submitted to the Protocol and Information Office prior to opening this study for patient entry.

The NCI will supply the following investigational drugs for this study:

NO DRUG SUPPLIED

If NCI is supplying drug(s), the protocol is being filed with the Food and Drug Administration as an amendment to the NCI sponsored IND(s). You may now order investigational drug supplies by submitting a typed Clinical Drug Request Form (NIH-986) to the Drug Management and Authorization Section, PMB, NCI, Executive Plaza North, Room 707, Bethesda, Maryland, 20892. Please limit your drug orders to an eight-week supply.

All correspondence and reviews relevant to the protocol will be maintained in our files and made a matter of record for the next review of your Group. A progress report on this study should appear in the Minutes of every future meeting of your Group until the study is completed, and we have been notified.

Sincerely,

Bruce D. Cheson, M.D.
Head, Medicine Section
Cancer Therapy Evaluation Program
Division of Cancer Treatment,
Diagnosis, and Centers
National Cancer Institute

Encl.

EXHIBIT 2

Southwest
Oncology Group
A National Clinical Research Group

September 1, 1998

TO: ALL SOUTHWEST ONCOLOGY GROUP, CCOP AND CGOP MEDICAL ONCOLOGISTS AND PATHOLOGISTS

FROM: Marilyn L. Slovak, Ph.D. and Wendy Stock, M.D.

RE: **S9719**, "Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence, Ancillary to **S9623**." Study Coordinators: Drs. M. Slovak, C. Willman, W. Stock, and K. Albain.

ANNOUNCEMENT

We would like to encourage your participation in a recently opened Southwest Oncology Group study, **S9719**, an ancillary study for patients enrolled on the Southwest Oncology Group intergroup adjuvant breast cancer study for women with 4-9 positive nodes, **S9623**. This is a study to evaluate the incidence of clonal hematopoiesis as a marker of genetic damage following dose intensive adjuvant therapy for breast cancer. This study was developed in response to increasing concerns about the rising incidence of therapy-related leukemias following dose intensive treatments for breast cancer.

Before randomization on the therapy protocol (**S9623**), all patients are required to have a bone marrow examination. At the time of this bone marrow, we are requesting a 2 ml aspirate. However, if this bone marrow sample cannot be obtained, patients may still be enrolled on the study. This is the only time that patients will be requested to donate bone marrow as part of either study (**S9719** or **S9623**) unless there is a later diagnosis of a secondary malignancy. All subsequent analyses will be performed on peripheral blood, with the exception of those women randomized to transplant, in which case a 2 ml apheresis sample is also requested at the time of stem cell collection. Blood will be collected before treatment, at apheresis, and at three and twelve months following the completion of protocol treatment when the patient returns for follow-up clinical visits.

Kits for sample collection and shipping are available and will be shipped to each institution by overnight mail. Alternately, kits may be obtained in advance and kept frozen until ready for use (see **S9719**, Section 4.0). Also, funds are available to offset mailing costs for samples sent to the University of New Mexico Cancer Center for this study. Please see Amendment #2 for the **S9719 Mailing Cost Reimbursement Form** and instructions.

We believe the results of this study will provide important insights into the effects of high dose therapy on hematopoietic stem cells and will identify whether certain dose-intensive drug regimens predispose patients to the development of a secondary leukemia. We look forward to your participation and would be happy to respond to any questions or concerns about the study.

cc: Stephanie J. Green, Ph.D.
Danika Lew, M.A
Diana Lowry
Camille White
Sarah Effert
Karin Rantala
Marjorie A. Godfrey

EXHIBIT 3

Southwest Oncology Group — A National Clinical Research Group

SWOG Cytogenetics Committee Office
City of Hope National Medical Center
1500 E. Duarte Road, NW Bldg., Room 2249
Duarte, CA 91010-0269
(626) 930-5385 (Office)
(626) 930-5390 (Fax)

February 16, 1998

Stacy K. Lewis, M.D.
Willford Hall U.S.A.F. Medical Center
BMT Program
SGHMMH
Lackland AFB, TX 78236

Dear Dr. Lewis:

We are writing to you to encourage the participation of your institution in a recently opened study, SWOG 9719, an ancillary study for patients enrolled on the intergroup adjuvant breast cancer study for women with 4-9 positive nodes, SWOG 9623. SWOG 9719 is a study to evaluate the incidence of clonal hematopoiesis as a marker of genetic damage following dose-intensive adjuvant therapy for breast cancer. This study was developed in response to increasing concerns about the rising incidence of therapy-related leukemias following dose-intensive treatments for breast cancer. The investigation is being funded through an independent grant to SWOG from the Department of Defense.

The requirements for samples from the patients are quite minimal and are outlined briefly below. Prior to randomization on the therapy protocol (S9623), all patients are required to have a bone marrow examination. At the time of this bone marrow, we are requesting a 2 ml aspirate. This is the only time that patients will be requested to donate bone marrow as part of either study (S9719 and S9623). All subsequent analyses will be performed on peripheral blood, with the exception of those women randomized to transplant, in which case a 2 ml apheresis sample is also requested at the time of stem cell collection. Blood and buccal mucosal brushings (a painless procedure done by brushing the buccal mucosa with a soft cytology brush) will be collected prior to treatment, at apheresis, and three and 12 months following completion of all treatment when the patient returns for follow-up clinical visits. Kits for sample collection and shipping are available (and will be shipped to you by overnight mail) from Dr. Cheryl Willman's laboratory at the University of New Mexico (see S9719 protocol, Section 4.0, Procedures/Sample Submission Requirements).

We believe the results of our study will provide important insights into the effects of high dose therapy on hematopoietic stem cells and help to identify whether certain dose-intensive drug regimens predispose patients to the development of a secondary leukemia.

We look forward to your participation in the study and would be happy to respond to any questions or concerns about the study.

Sincerely,

Marilyn L. Slovak, Ph.D., FACMG
Associate Research Scientist
City of Hope National Medical Center
Phone: 626-359-8111 x: 2313
Fax: 626-301-8877

Wendy Stock, M.D.
Assistant Professor of Medicine
University of Illinois at Chicago
Phone: 312-355-0840
Fax: 312-413-4131

EXHIBIT 4

Southwest Oncology Group Memo

To: Daniel Hayes, M.D.
Larry Norton, M.D.

From: Marilyn L. Slovak, Ph.D.
Wendy Stock, M.D.

CC: Gary Gilliland, M.D.
Stephen J. Forman, M.D.
E.J. Shpall, M.D.

Date: 05/07/98

Re: Invitation to participate : S9719, Clonal Hematopoiesis Protocol

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We are writing this letter to invite CALGB to participate in a recently opened study, S9719, an ancillary study for patients enrolled on the intergroup adjuvant breast cancer study for women with 4-9 positive nodes, S9623. S9719 is a study to evaluate the incidence of clonal hematopoiesis as a marker of genetic damage following dose intensive adjuvant therapy for breast cancer. This study was developed in response to increasing concerns about the rising incidence of therapy-related leukemias following dose intensive treatments for breast cancer. The investigation is funded through an independent grant to SWOG from the Department of Defense.

The requirements for samples from the patients are minimal and are outlined below. Before randomization on the therapy protocol (S9623), all patients are required to have a bone marrow examination. At the time of this bone marrow, we are requesting a 2-ml aspirate. This is the only time that patients will be requested to donate bone marrow as part of either study (S9719 and S9623). All subsequent analyses will be performed on peripheral blood, with the exception of those women randomized to transplant, in which case a 2 ml apheresis sample is also requested at the time of stem cell collection. Blood will be collected before treatment, at apheresis, and three and 12 months following the completion of all treatment when the patient returns for follow-up clinical visits. Kits for sample collection and shipping are available (and will be shipped to you by overnight mail or you may obtain the kits in advance and keep them frozen until ready for use) from Dr. Cheryl Willman's laboratory at the University of New Mexico (see S9719, Section 4.0, Procedures/Sample Submission Requirements).

We believe the results of this study will provide important insights into the effects of high dose therapy on hematopoietic stem cells and help to identify whether certain dose-intensive drug regimens predispose patients to the development of a secondary leukemia.

We hope you will consider this invitation to participate in this study. We would be happy to respond to any questions or concerns about the study.

Marilyn L. Slovak, Ph.D., FACMG
Associate Research Scientist
City of Hope National Medical Center
Phone: 626/359-8111 ext 2313
FAX: 626/301-8877
Email: mslovak@smtplink.coh.org

Wendy Stock, M.D.
Assistant Professor of Medicine
University of Illinois at Chicago
Phone: 312/355-0840
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Email: wstock@uic.edu

EXHIBIT 5

Southwest
Oncology Group
A National Clinical Research Group

December 1, 1997

TO: ALL SOUTHWEST ONCOLOGY GROUP, CCOP AND CGOP MEDICAL ONCOLOGISTS AND PATHOLOGISTS

FROM: Tamra N. Oner/Protocol Coordinator

RE: S9719, "Clonal Hematopoiesis as a Marker of Genetic Damage Following Adjuvant Chemotherapy for Breast Cancer: Pilot Study to Evaluate Incidence, Ancillary to S9623." Study Coordinators: Drs. M. Slovak, C. Willman, W. Stock, and K. Albain.

REVISION #1

Section V of the Model Consent Form has been modified to clarify that the local institutions will be responsible for any necessary medical care for physical injury or disease which is determined to be the proximate (or direct) result of participation in this study. This section has been bolded, and is required to be used in its entirety.

A replacement page is enclosed for page 17. Please insert this notice and page 17 to your copy of the protocol.

This memorandum serves to inform the Southwest Oncology Group Statistical Center, and the NCI.

cc: Stephanie Green, Ph.D.
Danika Lew, M.A
Patricia Waldman
Diana Lowry
Camille White
Sarah Effert
Marjorie Godfrey