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TITLE: Chemotherapy of Late Stage Breast Cancer Targeted Towards
Cell Cycle Regulatory Components

PRINCIPAL INVESTIGATOR: Robert A. Sclafani, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado, HSC
Denver, Colorado 80262

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13. ABSTRACT (Maximum 200 Words) Our original goal was to target cell cycle regulatory components as a potential therapy for breast cancer. The target p16 is a tumor suppressor and a CDK inhibitor and is inactivated by gene methylation. The drug 5-aza-2'deoxyctidine (5-Aza-CdR), an inhibitor of DNA methylation, is targeted for re-expression of a repressed p16 in late stage breast cancer patients. Re-activation of the p16 gene in cells in which the gene is methylated will restore normal growth control and be efficacious in treatment of breast cancer. A mouse model for the action of the drug was developed and used to test the efficacy of the drug. Our findings have not been conclusive because of the inherent toxicity of this drug. Furthermore, using a blood test we developed for the detection of p16 methylation, the number of patients suitable for a 5-Aza-CdR clinical trial was found to be very low. A phase II clinical trial with a second drug, Bryostat-1, which regulates the p21 CDK inhibitor, was terminated due to dismal accrual rates. A new potential target that we are exploring is the Cdc7 gene, which regulates the cell cycle and mutagenesis and is up-regulated in many breast cancers.

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Introduction

During the past decade a very large increase in our knowledge of biological mechanisms regulating progression of cells through the cell division cycle has taken place. Together with the development of this knowledge, it has been shown by work in many laboratories that essentially all cancer cells have one or more defects in the components known to regulate cell cycle progression. For example, our recently completed studies of cell cycle regulatory defects in breast cancer cells, carried out with a grant from the Army Breast Cancer Research Program, showed that loss of expression of the cyclin-dependent kinase inhibitor p16, sometimes accompanied by overexpression of cyclin D1, is a common defect in breast cancer cells. These findings, plus the large amount of work carried out by others, presented a new potential target for cancer chemotherapy. Our proposal to exploit such targets for the chemotherapy of breast cancer is the basis for the current Clinical Translational Research Grant. We proposed to explore two drugs known or expected to cause changes in the expression of cell cycle regulatory components as potential chemotherapeutic agents in the treatment of late stage breast cancer. Bryostatins, shown by Kraft and coworkers to cause increases in the expression of the cyclin-dependent kinase inhibitor p21, was chosen as an agent to be tested in a phase II clinical trial. 5,6-dihydro-5-azacytidine, a DNA methylation inhibitor with less toxicity than the commonly studied 5-aza-2'-deoxycytidine, was chosen for pre-clinical studies directed towards eventually implementing a phase II clinical trial of that drug. DNA methylase inhibitors have been shown to increase the expression of p16 protein in cells where lack of expression is due to methylation of the p16 gene. Our animal studies have revealed potential caveats about the use of 5-aza-2'-deoxycytidine in clinical trials. Our human studies have shown that the number of patients who present with methylated p16 is low, so it is not clear if this drug would be useful. Therefore, we have begun to look at a different cell cycle regulator, the human Cdc7 gene, which we find is overexpressed in many human breast cancer cell lines. The results of the fourth year of the grant are reported here.

Body of Report

Materials and Methods

Breast cancer cell lines and tumor material

12 breast cancer cell lines and one normal mammary epithelial cell line (MCF-10A) were obtained from the University of Colorado Tissue Culture Core Facility and the American Type Culture Collection (listed in **Table 1**). We have characterized them previously for cell cycle regulatory molecules and currently results for Cdc7 are included in the Appendix (**Figure 1** and **Table 1**).

Antibodies

The anti-p16 was obtained from Oncogene. The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad. Antibodies for human Cdc7 were obtained from Dr. Jiang at the Burnham institute (San Diego, CA). We are currently purifying and characterizing chicken anti-Cdc7 antibodies that we have had made commercially (Aves Labs). We propose to use these new antibodies in immunohistochemical (IHC) studies of human tumors.

Protein extraction and western blot analysis

Cells were harvested and washed in PBS then resuspended in Laemmli sample buffer (Laemmli, 1970). After boiling for 4 minutes, the extracts were sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C.

Approximately 100 mg of each protein extract were subjected to SDS/PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Schleicher and Schuell) for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading, and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Isolation of DNA from cell lines and blood plasma

Cell line DNA was isolated by incubating cells at 55°C in lysis buffer (10 mM Tris pH 8.0, 2.0 mM EDTA pH 8.0, 10 mM NaCl, 5% SDS) containing 1 mg/ml Proteinase K. The samples were then subjected to two phenol-chloroform extractions and one chloroform:isoamylalcohol (24:1) extraction followed by ethanol precipitation.

To isolate plasma DNA, 10 ml plasma were first heated to 99°C for 5 minutes then centrifuged at 14 K rpm. The clear supernatant was incubated overnight in one-tenth volume of 20 mg/ml Proteinase K (in double distilled water) and one-tenth volume AL buffer (QIamp Blood Kit, Qiagen Inc., Hilden, Germany) after which the DNA was purified on QIAamp columns (QIamp Blood Kit, Qiagen Inc., Hilden, Germany) according to the 'Blood and Body Fluids protocol'. The DNA was eluted from the column with 200ul double distilled water.

DNA analysis by Methylation-specific PCR (MSP)

Two micrograms of cell line DNA or one-fourth of the total plasma DNA sample was modified with sodium bisulfite using a modified method of (Herman et al., 1996) kindly sent to us by S. Belinsky (University of New Mexico). The DNA was then precipitated with ammonium acetate (3M final concentration) and two volumes of ethanol. The resulting templates were subjected to a nested, two-stage PCR. This two-step PCR approach improves the sensitivity to detect methylated alleles by >50 fold over the original method (Palminsano et al. 2000). Templates were subjected to stage-1 PCR with primers designed from the promoter of the p16 gene (Palminsano et al. 2000). A 50µl reaction mixture contained a final concentration of 10µM of each oligonucleotide, PCR buffer supplied by Qiagen Hotstar Taq DNA polymerase kit (10x Buffer= Tris.Cl, KCl, (NH₄)₂SO₄, 15mM MgCl, pH8.7 note:Qiagen does not describe specific concentrations for its buffer); 200 mM dNTPs; and 1.25 units Qiagen Hotstar Taq DNA polymerase. The DNA was subjected to 40 cycles of amplification consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and elongation for 30 seconds at 72°C, followed by a final elongation step of 10 minutes at 72°C. The stage-1 PCR products were diluted 50 fold and 5µl was subjected to a stage-2 PCR. A 20µl reaction mixture contained a final concentration of 10µM of each oligonucleotide, PCR Master mix supplied by Qiagen Hotstar Taq DNA polymerase kit (Tris.Cl, KCl,(NH₄)₂SO₄, 15mM MgCl, ph8.7, 200 mM dNTPs); and 0.5 units Qiagen Hotstar Taq DNA polymerase. The DNA was subjected to 40 cycles of amplification consisting of denaturation for 15 seconds at 94°C, annealing for 15 seconds at 68°C for unmethylated-specific and methylated-specific oligonucleotides (Herman et al. 1996), and elongation for 15 seconds at 72°C, followed by a final elongation step of 10 minutes at 72°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with UV light. Multiple steps were required to prevent cross-contamination of PCR samples. Some of these steps included: (1) filter pipette tips were used for all PCR reactions, (2) filter sterilized H₂O was used instead of autoclaved H₂O, (3) we reduced the number of pipette manipulations by creating a "mastermix" of all the components required for PCR except for the DNA template and Taq polymerase, (4) "mastermix " components were stored in a freezer away from DNA templates, and (5) DNA template was added to reactions as the final step in setting up the reactions.

Tumorigenicity Studies of Human Breast Cancer Cell Lines in Athymic Nude mice.

The growth of human breast cancer cell line T47D-DE in nude mice was tested with xenographs in animals treated or not treated by repeated intraperitoneal administration of the drug 5-Aza-CdR or with no drug (PBS control), by injecting subcutaneously on the right and left flank of each animal with a suspension (volume 0.4ml) of growth factor reduced Matrigel (BD Biosciences) and living human breast cancer cells that were grown previously in tissue culture. The cell line T47D-DE was tested in mice that have been ovariectomized and hormone replacement accomplished via insertion of a time-release (60 day) estrogen (17Beta-estradiol, Innovative Research of America) hormone release pellet inserted at the base of the mouse's neck. The mice were handled exclusively in the UCHSC Animal Resource center, using sterile equipment, clothing, and facilities. They were maintained there after surgery and injections, and inspected weekly to measure the development of and size of any tumors. After a period of not more than 4 weeks we injected the mice three times a week at a dose of 3mg/kg of the drug 5-Aza-CdR or sterile PBS (control) for an additional 4 weeks. The mice were sacrificed humanely by CO₂ narcosis and any tumor removed for molecular and pathological analysis.

Relationship to Statement of Work

Tasks 1, 2, and 3. To implement and evaluate a Phase II clinical trial designed to test the chemotherapeutic efficacy of bryostatin 1 in Stage IV breast cancer patients who have failed high dose chemotherapy.

Bryostatin-1 Trial for Breast Cancer

This phase 2 trial was terminated by the NCI in March of 2002. The main reason was the dismal accrual rate. Dr. Kraft of this grant is the PI for this trial and he has communicated these results to Dr. Adriene King of AMDEX corp., who is the Human Subjects Protection Specialist for the USAMRMC (see Memo MCMR-RCQ (70-1n).

Task 4. To determine the effects of DHAC and 5aza-2'deoxyctidine on p16 levels and on growth and tumor formation by breast cancer cells.

As described in previous progress reports, DHAC proved to be ineffective as a DNA methylation inhibitor capable of inducing expression of p16 in breast cancer cell lines. Thus, we turned to the more widely studied methylation inhibitor 5aza-2'deoxyctidine (5-Aza-CdR). In the previous progress report we had demonstrated that 5-Aza-CdR causes demethylation of p16 DNA and expression of p16 protein in several breast cancer cell lines including T47D-DE and T47D-CO, with concomitant loss of growth of these cells in soft agar. We also reported on the effect of the drug 5-Aza-CdR in nude mice following tumor implantation of breast cancer cells. Our results also show

that 5-Aza-CdR will inhibit the growth of MCF-7 cells in soft agar. In contrast, MCF-7 cells do not have methylated p16 but are deleted for p16. This implies that the effect of 5-Aza-CdR is not specific to p16. Thus, 5-Aza-CdR must be inhibiting the growth of MCF-7 cells by affecting genes other than p16. We confirmed this result by showing that overexpression of p16 arrests T47D cells in G1 of the cell cycle, but that treatment with 5-Aza-CdR does not.

In contrast, our initial animal studies were encouraging in that tumors were smaller in mice treated with 5-Aza-CdR. However, we have seen high toxicity (>80%) for 5-Aza-CdR which has frustrated out attempts to complete these experiments with statistically significant data. All in all, the combination of the lack of specificity and the toxicity do not bode well for the use of 5-Aza-CdR in human patients.

Task 5. To test the effects of combination of DHAC, 5-Aza-CdR and bryostatin 1 on growth and tumor formation by breast cancer cells. Months 24-48.

Again, DHAC was ineffective in vitro so it was not used. The bryostatin 1 trial has been closed (See Tasks 1, 2 and 3). 5-Aza-CdR was effective but toxic and not specific (See Task 4).

Task 6. To design a Phase II clinical trial of 5-Aza-CdR.

We recommend against this trial because 5-Aza-CdR is toxic and not specific (See Task 4).

Task 7. To implement a Phase II clinical trial of 5-Aza-CdR in breast cancer patients who have failed high dose chemotherapy and whose tumors contain methylated p16 DNA.

We recommend against this trial because 5-Aza-CdR is toxic and not specific (See Task 4).

Task 8. To evaluate the outcome of 5-Aza-CdR chemotherapy in terms of effects on tumor response as well as on methylation of tumor p16 DNA, expression of p16, cdk4/cdk6 kinase activity and phosphorylation state of Rb protein.

We recommend against this trial because 5-Aza-CdR is toxic and not specific (See Task 4).

Task 9. To develop methodology for determining the p16 methylation status of breast cancer patients by measurements on plasma DNA, and to employ

this methodology for the selection of patients for clinical trials of 5-Aza-CdR in breast cancer.

Although we recommend against a 5-Aza-CdR clinical trial, we believe that knowledge of the fraction of breast cancer patients with methylated p16 is still useful to know. We have developed two protocols to evaluate the methylation status of the p16 gene in human samples. These protocols involve a unique assay of methylated p16 DNA done using DNA that is free in the blood of patients. The first protocol #00-848 enables us to examine blood from 100 patients that are known to have metastatic breast cancer. Patients with known metastatic disease have a single sample of blood drawn and the plasma frozen for later analysis. Statistical analysis demonstrates that p16 should be methylated in 30% of the tumors according to the published reports (Herman et al, 1995). This number of samples should give us sufficient numbers of samples to demonstrate whether this blood test is sufficiently sensitive to detect p16 DNA in the blood. The second protocol will enable us to correlate the p16 methylation in samples taken at the time of breast biopsy or surgery with the blood samples. DNA is extracted from the tissue and the blood and then analyzed by sensitive PCR for the methylation of p16.

The second protocol #00-849 enables us to correlate the p16 methylation in samples taken at the time of breast biopsy or surgery with the blood samples. DNA is extracted from the tissue and the blood and then analyzed by sensitive PCR for the methylation of p16. So far only one patient out of 31 has been shown to demonstrate a methylated p16 allele. This includes ten new samples we have analyzed in the last period.

We have had to re-write both protocols after being contacted in March of 2002 by Dr. Adriene King of AMDEX corp., who is the Human Subjects Protection Specialist for the USAMRMC. Both protocols have been re-written (See Appendix) and should be approved so we can begin accruing patients again.

Task 10 (New Task). To measure the level of Cdc7 protein in human breast cancer cell lines and in breast cancer patient samples.

We have found that 11/12 breast cancer cell lines overexpress Cdc7 protein (Fig. 1 in the Appendix). In contrast, no overexpression is seen in the normal epithelial cell lines MCF-10A and MCF-12A. We find a correlation between Cdc7 overexpression and a deregulated G1 to S phase in these cell lines (Table 1 in the Appendix). We believe Cdc7 represents an important biomarker in cancer as shown by previous results from my laboratory (Hess et al., 1998; Sciafani, 2000). Cdc7 is known to effect the regulation of both DNA replication and mutagenesis, which are both altered in cancer cells (Sciafani, 2000). Our hypothesis is that Cdc7 protein levels could be predictive of poor prognosis in breast cancer. If this new Task is approved, we plan to further investigate the level of Cdc7 in human breast cancer cell lines and in patient samples. We would seek approval from both the USAMRMC and our internal IRB for the human studies.

Key Research Accomplishments

- We have shown that the inhibitory effects of 5-AzaCdr on breast cancer cells are not specific to cell lines containing a methylated p16 gene.
- We have developed a model that will allow us to study human breast cancer tumors in nude mice. We have used this model system to test the efficacy of methylation inhibitors such as 5-Aza-CdR. Our results show that 5-Aza-CdR is toxic in mice and we recommend against using it in patients.
- We have found that methylated p16 sequences are readily detectable in DNA isolated from plasma of patients. However, a low percentage (3%) of patients present with this defect.
- We have found that Cdc7 protein represents a potential biomarker in breast cancer in that a majority of breast cancer lines have overexpressed Cdc7 protein. We show a high level of positive correlation of Cdc7 overexpression with de-regulation of the G1 to S phase transition of the cell cycle.

Reportable Outcomes

There have been no reportable outcomes to date.

Conclusions

Our previous studies of the effects of 5Aza-Cdr on p16 gene methylation and p16 protein expression in breast cancer cell lines have shown that this drug effectively blocks p16 gene methylation and induces p16 protein production. 5-Aza-Cdr also blocks anchorage-independent growth of breast cancer cell lines, indicating that treatment with this drug abrogates the tumorigenic properties of these cells. However, control experiments employing breast cancer cell lines with deleted p16 genes also are inhibited by 5-Aza-Cdr, indicating that effects on gene expression other than the induction of p16 may contribute to this effect.

We have added to our previous data and have showed that our animal model for re-activation of p16 by 5-Aza-Cdr shows high toxicity. We believe that these combined

results raise serious concerns about this type of chemotherapy and we recommend against it.

Our development of a protocol for detecting methylated p16 sequences in plasma DNA from breast cancer patients provides a potential non-invasive procedure for determining the p16 methylation status of breast cancer patients. We have used this procedure to demonstrate that the amount of late stage breast cancer patients with a methylated p16 gene is much lower (3%) than expected according the literature (30%).

We obtained IRB for the Bryostatins-1 phase 2 clinical trial. However, this trial was terminated by the NCI in March of 2002. The main reason was the dismal accrual rate.

"So What" Section

Our results have provided important caveats to cancer chemotherapy in which treatment with a methylation inhibitor, 5-Aza-Cdr, is proposed to activate a repressed, methylated tumor suppressor gene. A combination of a lack of specificity and toxicity makes this type of chemotherapy untenable. Furthermore, our clinical studies have shown that the number of patients with this type of lesion may be much lower than originally thought.

We hope that our new studies with Cdc7 protein may represent a potential biomarker in breast cancer, be important for patient prognosis and may represent a potential target for therapy.

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Appendix

-Figure 1

-Table 1

-Revised Clinical Protocols and Patient Consent Forms:

#00-848: "Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay"

#00-849: "Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients"

Figure 1. Immunoblot of Cdc7 protein in breast cancer cell lines

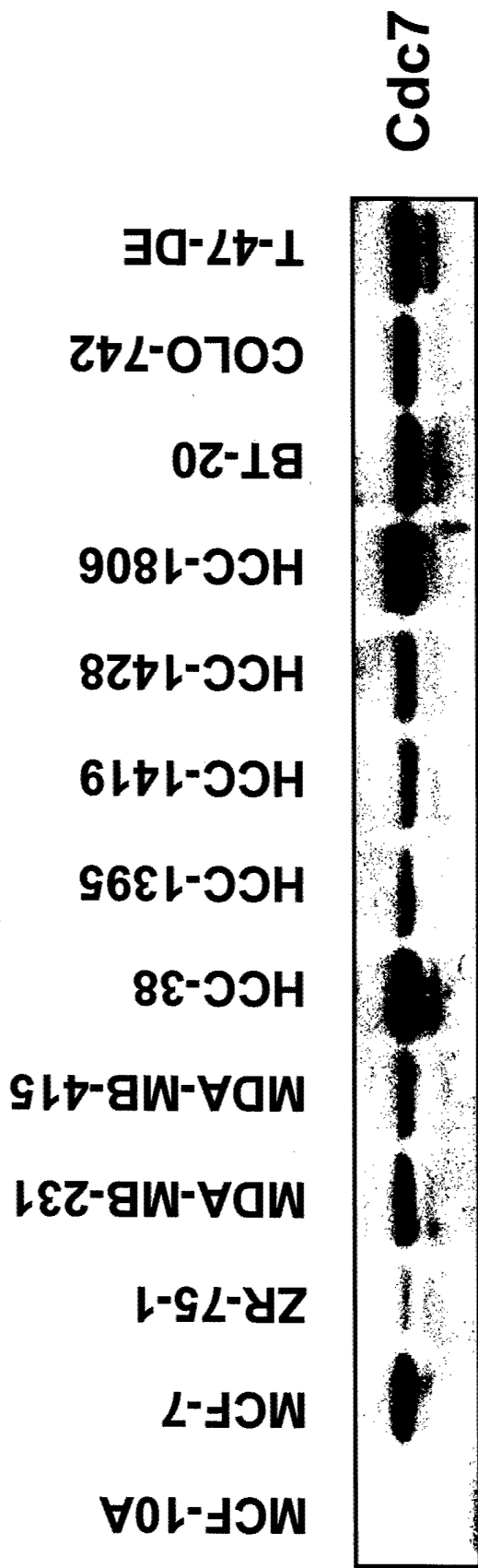


Table 1. Cell Cycle Proteins in Breast Cancer Cells

Cell Line	Rb	cycD1	p16	Cdc7
MCF-12A	+	+	+	Low
MDA-MB-231	-	-	-	High
COLO591	-	-	-	High
COLO742	+	High	-	High
MCF-7	+	High	-	High
T47D	+	High	-	High
ZR75.1	+	+	-	Low
MDA-MB-415	+	High	+	High



Division of Medical Oncology
4200 East Ninth Avenue, B171
Denver, Colorado 80262
Phone: 303-315-8802
Fax: 303-315-8825

August 26, 2002

Dr. Adriene D. King
Human Subjects Protection Specialist
Amdex Corporation

Dear Ms. King,

I decided to FedEx this package in order for you to receive it as quickly as possible.

Enclosed please find the following:

A copy of the letter previously sent to you which list the following items that were enclosed with that letter:

- a) Dr. Sclafani's C.V.
- b) A copy of the consent form for standard medical procedures.
- c) A copy of the authorization to release medical information.
- d) A copy of protocol 00-849 with the suggested revisions.
- e) A copy of the consent forms for both 00-848 and 00-849 with revisions.

I have also included the following:

1. A copy of protocol 00-848
2. COMIRB Certificates of Approval for both protocol 00-848 and 00-849.

Please contact me if there is anything else you require.

Sincerely,

Laura D. Casias
Division of Medical Oncology
University of Colorado Health Sciences Center
Phone 303-315-8802
Fax 303-315-8825
Email laura.casias@uchsc.edu

UNIVERSITY OF COLORADO HOSPITAL 4206 E. Ninth Avenue Denver, CO 80262 CONSENT TO MEDICAL PROCEDURE (SURGERY, DIAGNOSTIC, THERAPEUTIC, BLOOD, SEDATION, ANESTHESIA) <i>Not to be used for investigational procedures</i>	HOSPITAL # PATIENT NAME Date:
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III. Blood:

I understand that I may need a transfusion of blood or blood products. While the blood has been screened for hepatitis, HIV and other abnormal antibodies, there is a remote risk of contracting these or other infectious diseases from the transfusion. Alternatives to transfusion and the risks, including death, have been explained to me.

I Accept (Patient Initial) I Refuse (Patient Initial) (see below)

IF BLOOD OR BLOOD PRODUCT ADMINISTRATION IS REFUSED

- 1.) REFERENCE "BLOOD REFUSAL POLICY"
- 2.) "REFUSAL TO PERMIT ADMINISTRATION OF BLOOD OR BLOOD COMPONENTS " MUST BE COMPLETE
- 3.) A "NO BLOOD" ID BAND MUST BE APPLIED TO THE PATIENT IMMEDIATELY

IV. Sedation:

Non applicable (Physician Initial)

My sedation will be given under the supervision of the provider performing my examination/procedure. Medication will be given to provide sedation and some pain relief. Rare complications include deep sedation, requiring placement of a breathing tube, loss of consciousness, pain during the examination, allergic reaction cardiac arrest, or other organ damage. Common side effects of sedation include mood alteration, (including but not limited to depression, sleepiness, confusion, excitability and loss of memory). Alternatives, benefits, and risks have been explained to me. I realize that I should not drive or operate heavy machinery until the day following the procedure and that I will need some one to drive me home the day of the procedure. I have been given the opportunity to ask questions concerning sedation and such questions have been answered to my satisfaction.

V. Specimen Disposition:

Non applicable (Physician Initial)

I authorize this health care facility to examine, photograph or preserve for teaching or research purposes, or to otherwise dispose of the tissue, limbs, organs or foreign objects resulting from the operation or procedure authorized above.

VI. Photography or Medical Images:

Non applicable (Physician Initial)

I authorize the above provider to take photographs or medical images of the procedure specified above and to use the pictures for hospital records, teaching and in medical and scientific publications.

VII. Non-Medical Center Observers In The Procedure Room:

Non Applicable (Physician Initial)

I authorize the above provider to have _____ present in the operating room as an observer during my surgery.

UNIVERSITY OF COLORADO HOSPITAL
 4200 E. Ninth Avenue
 Denver, CO 80262

**CONSENT TO MEDICAL PROCEDURE
 (SURGERY, DIAGNOSTIC, THERAPEUTIC,
 BLOOD, SEDATION, ANESTHESIA)**

Not to be used for investigational procedures

HOSPITAL #

PATIENT NAME

Date:

VIII. Disposition of Fetal Tissue: Non Applicable (Physician Initial)

I understand that if I wish to retrieve embryo, fetus, or other pregnancy tissue for private funeral services, I must indicate such wishes on this consent form. If no preference is indicated, the pregnancy tissue will be disposed of by the University of Colorado Hospital.

- Please dispose of embryo, fetus, or pregnancy tissue. (Initial)
- I wish to retrieve the embryo, fetus, or other pregnancy tissue for private funeral services. I will contact Decedent Affairs within 24 hours of the procedure or the Hospital will dispose of pregnancy tissue. (Initial)

IX. Patient Consent:

I hereby give my consent to the above.

Exceptions: _____

 Signature of Patient, Legal Guardian,
 or Patient's Representative

 Relationship to Patient

 Print Name of Signer

 Date Time

X. Physician Declaration:

I believe this patient is an appropriate candidate to undergo the planned operation / procedure. I have explained the risks and alternatives of the operation/procedure to the patient and have answered all the patient's questions. To the best of my knowledge, the patient has been adequately informed, understands, and has consented to the proposed operation or procedure.

 Signature of Informing Physician

 Date Time

XI. Certification of Emergency by Physician:

I have determined that immediate intervention is necessary to preserve life, or prevent serious impairment of physical or mental health. The patient's current mental or physical condition prevents obtaining expressed consent. The Patient's Representative is not available.

Brief statement on condition and plan of care

 Physician certifying emergency

UNIVERSITY OF COLORADO HOSPITAL
4200 E. Ninth Avenue
Denver, CO 80262

**CONSENT TO MEDICAL PROCEDURE
(SURGERY, DIAGNOSTIC, THERAPEUTIC,
BLOOD, SEDATION, ANESTHESIA)**

Not to be used for investigational procedures

HOSPITAL #

PATIENT NAME

Date:

XII. Request for And Consent to Anesthesia Service:

My anesthetic care will be provided by or under the supervision of a faculty anesthesiologist. Anesthetic techniques recommended for my planned procedures are: _____

Anesthetics have rare complications such as awareness during surgery, allergic reactions, cardiac arrest, nerve damage, brain damage, paralysis, other organ damage or death. Common side effects of general anesthesia include sore or scratchy throat, nausea and vomiting, muscle or joint pains or headaches. The risks of regional anesthesia such as epidural or spinal blocks include pain at the needle site, discomfort during the operation, or nerve and/or spinal damage. If the regional anesthesia fails, another type of anesthesia, usually general anesthesia, is substituted. I have been given the opportunity to ask question concerning the recommended anesthesia and such questions have been answered to my satisfaction. I consent to the recommended anesthetic procedures except _____

Signature of Patient, Legal Guardian,
or Patient's Representative

Relationship to Patient

Print Name of Signer

Date

Time

Physician Declaration for Anesthesia:

I believe this patient is an appropriate candidate to undergo the planned anesthesia. I have explained the risks and choices of anesthesia to the patient and have answered all the patient's questions. To the best of my knowledge, the patient has been adequately informed, understands, and has consented to the proposed anesthesia.

Signature of Informing Anesthesiologist

Date

Time

UNIVERSITY HOSPITAL
4200 East Ninth Avenue, Denver, Colorado 80262

HOSPITAL NUMBER:

PATIENT NAME:

BIRTHDATE:

AUTHORIZATION TO REQUEST/RELEASE MEDICAL INFORMATION

(The execution of this form does not authorize the release of information other than that specifically described below)

TO: (Print/type name & address of doctor or health care facility)

RELEASE TO: (Name, address of organization, agency, or individual to whom information is to be released)

RETURN ENCLOSED AUTHORIZATION WITH YOUR INFORMATION

I request and authorize the above-named doctor or health care provider to release the information specified below to the organization, agency or individual named on this request.

INFORMATION REQUESTED:

- Copy of history & physical, discharge summary, and operative reports
- Copy of outpatient & E.R. admissions
- Copy of complete hospital record

X-ray films to be sent to: _____

Other (Specify) _____

I understand that information to be released may include information regarding the following condition(s):

- Drug abuse, if any
- Alcoholism or alcohol abuse, if any
- Autoimmune Deficiency (AIDS)
- Psychological or psychiatric conditions, if any

If the information to be released pertains to the diagnosis and treatment of alcoholism and/or drug abuse, I understand that the confidentiality of the information is protected by Federal Law.

Purpose(s) or need for which information is to be used:

- Continuity of medical care
- Damage or claim evaluation
- Other (Specify) _____

AUTHORIZATION - I certify that this request has been made voluntarily and that the information given above is accurate to the best of my knowledge. I understand that I may revoke this authorization at any time, except to the extent that action has already been taken to comply with it. I understand that unless specified below this consent will expire 180 days from the date of signature. _____ I hereby release the health care provider from any liability which may result from furnishing the information requested as authorized in this release. The health provider cannot be responsible for misuse of this information disclosed pursuant to this release.

DATE _____

SIGNATURE OF PATIENT _____

PERSON AUTHORIZED TO SIGN FOR PATIENT _____

ADDRESS _____

RELATIONSHIP OF CONSENTING PARTY TO PATIENT _____

CITY, STATE, ZIP CODE _____

PHONE _____

PATIENT'S ACKNOWLEDGEMENT OF ACCESS TO MEDICAL RECORDS

I hereby acknowledge that I/parent/guardian, or the designated representative have inspected _____ received _____ photocopies of the medical records of inpatient and/or outpatient treatment at University Hospital of the above named patient.

DATE _____ SIGNATURE _____
(Patient/Parent/Guardian/Designated Representative)

DATE _____ WITNESS SIGNATURE _____

Project Title: Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients.

Investigators: Andrew S. Kraft, MD, Christina A. Finlayson, MD, Robert Sclafani, Ph.D. and Thomas A. Langan, Ph.D.

COMIRB# 00-849

Date: August 28th, 2000

Background/Rationale

The importance of defects in the Rb pathway in the development of cancer is indicated by the fact that almost all cancer cells are defective in some aspect of its regulation (1-5). This near universal prevalence of defects in components of the Rb pathway suggests that overcoming normal cell cycle regulation at this point is necessary condition for the development of malignancy (5). These defects include loss of expression of Rb (6,7), overexpression of cyclin D1 (8), and loss of expression of p16 inhibitor protein, whose role as a tumor suppressor is now well documented (9). In particular, the importance of p16 in tumor suppression is seen in that homozygous p16 deletions in mice result in spontaneous development of multiple tumor types (10).

Recognition of the importance of p16 as a tumor suppressor comes in part from the recent discovery that gene methylation, in addition to homozygous deletion or loss of heterozygosity seen for other tumor suppressor genes is a major mechanism for inactivation of p16 gene in all common human cancers (11,12). In primary breast tumors, which only rarely show homozygous p16 deletions or point mutations (13,14), 31% of tumors were found to contain p16 genes inactivated by methylation (11). We hypothesize that p16 gene methylation is responsible for uncontrolled cellular proliferation in many breast tumors, and that agents that increase this protein will prevent the growth of cancer. For example, DHAC, an NCI-produced (NSC #264880) methylation inhibitor has been tested in phase I and phase II clinical trials.

It has been known for several years that the plasma component of circulating blood contains tiny quantities of free DNA. The concentration of DNA in the plasma of healthy individuals is approximately 14 ng/ml DNA. This level increases significantly in individuals diagnosed with different types of cancer to approximately 180 ng/ml.

The ability to detect tumor-specific molecular defects in the circulating blood would obviate the requirement for tumor biopsy material thus providing a more efficient and noninvasive means of screening for a vast array of molecular aberrations. Our objective is to identify women whose tumors contain methylated p16 genes. We propose to determine the feasibility of assaying plasma DNA for the presence of methylated p16 alleles in women diagnosed with breast cancer. DNA will be extracted from circulating blood plasma then assayed for p16 promoter methylation status by methylation-specific

PCR (MSP)3. The normal breast tissue is expected only to contain unmethylated p16 whereas the tumor tissue may contain either unmethylated or methylated p16. If the tumor DNA contains methylated p16 alleles, we should also expect to see methylated p16 in the plasma DNA in addition to normal cell-specific unmethylated p16.

If it is possible to determine which patients have methylated p16 it will be possible *in the future* to test specific agents to inhibit this methylation. These agents should increase p16 protein levels in breast tumors and inhibit tumor growth.

Hypothesis

We hypothesize that there will be a strict correlation between the occurrence of methylated p16 in tumor samples and the blood of patients. This result would validate the use of a PCR based assay method to detect methylated p16 gene in the blood of breast cancer patients. This test will replace the necessity of obtaining breast cancer tissue to measure the methylation of this gene. This assay may have uses both in designing treatment strategies and in the management of breast cancer patients.

Purpose

The data from this study will determine the correlation between breast tissue methylation of the p16 gene and the blood of patients evaluated using a PCR based blood test. Since 30% of breast cancer patients are expected to have methylated p16 DNA in their tumors, the data collected in this study will evaluate the sensitivity and specificity of this blood test.

Methods

Women being evaluated in breast cancer clinic who are found to have breast cancer metastatic disease will be asked to sign a consent allowing us to obtain a blood sample (20 ml) prior to operation or breast biopsy. A small amount of tissue from the surgery that is not needed for pathology will be analyzed in the laboratory. The normal tissue (5-10mg) will be collected as part of the clinical procedure to remove the cancerous tissue. All data concerning the extent of disease and disease progression will be kept confidential. The patient will be identified by number only and the data will be kept in locked files.

- Isolation of DNA from blood plasma

Using 10 ml plasma (from approximately 20 ml whole blood collected in EDTA containing tubes), we have optimized the following isolation procedure to yield sufficient DNA for an initial MSP assay plus three further confirmatory reactions.

The 10 ml plasma sample (which may be frozen at -80°C prior to analysis) is first heated at 99°C for 5 minutes then centrifuged at high speed to allow recovery of the clear, DNA containing, supernatant. Following an overnight incubation with proteinase K the DNA is purified on QIAamp columns (QIamp Blood Kit; Qiagen Inc., Hilden, Germany) according to the Blood and Body Fluids protocol.

- Isolation of DNA from normal and tumor tissues

5-10 mg finely minced tissues are incubated 55°C in lysis buffer containing proteinase K until the tissue is completely lysed. The DNA is then purified on QIAamp columns (QIAamp Tissue Kit; Qiagen Inc., Hilden, Germany) according to the Tissue protocol.

- Methylation-specific PCR analysis of plasma or tumor samples.

Two micrograms of tissue DNA or one-fourth of the total plasma DNA sample is then used for the MSP assay. The DNA is first modified overnight with sodium bisulfite which converts only unmethylated cytosines to uracil. Following purification on Wizard columns (Promega, Inc.), the DNA is precipitated with ammonium acetate and ethanol. One-tenth of the resulting DNA is then subjected to PCR analysis using oligonucleotides designed from the promoter of the p16 gene that are specific for wild type (unmodified), methylated and unmethylated DNA sequences.

We have performed MSP analysis of multiple independently-isolated normal plasma DNA preparations and, as expected, shown amplification using the unmethylated DNA-specific oligonucleotides only. Furthermore, we have titrated cell line DNA containing methylated p16 alleles into plasma samples prior to isolation of plasma DNA and shown that we can successfully amplify as little as 12.5 ng methylated DNA in addition to the unmethylated normal plasma DNA using this method. Because the normal plasma DNA contains only unmethylated DNA, this system will enable us to identify tumor-specific methylated DNA in the plasma of breast cancer patients.

Observations

- 1- We will plan to determine that the above assay could be done on actual patient material.
- 2- Since approximately 30% of the tumors are positive to p16 methylation, whether the results of this blood assay correlates with the tumor samples.
- 3- We would plan to correlate the stage and extent of disease with the number of positive assays in the blood.

Inclusion criteria

Woman ages 18-72 years that are known to have breast cancer and are set to undergo biopsy or operation as determined by x-ray and clinical history. Women

seen and treated in the University of Colorado Cancer Center breast cancer clinic will be invited to participate in this study. No flyers or ads will be used.

Exclusion criteria

- 1- Patients deemed not competent to make their own decision or do not have a guardian willing to enroll the patient into the study.
- 2- Patients with a previous history of another cancer.

Sample Size

We will be able to establish a confidence interval of at least ± 0.1 on the percent agreement of the paired tissue and blood samples with a paired sample size of 100. This estimate of the level of agreement can be established regardless of the sensitivity or specificity of the assay in either case.

Estimated Duration of Study

The estimated duration of the study is 1 year. If the samples are collected more quickly the study will be terminated.

Examinations and evaluations

Other than the drawing of 20-30 cc of blood no further examination of the patient will be done. The extent of breast cancer will be noted from the chart.

Drugs, devices or instruments

No novel drugs devices or instruments are involved in this study.

Data analysis - Data analysis will be carried out by Dr. Jim Murphy in biostatistics.

Data Security

All electronic data will be secured in protected files. All paperwork will be secured under lock and key. Each patient sample will be identified with a code that will be secured as noted above. This code will be used on all generated data and throughout all discussions of these results. Only the Principal investigators will have access to the storage files containing the code.

Changes from Usual Treatment

There will be no changes in treatment. All participants will receive therapy deemed as standard of care by their primary oncologist. Because of the extreme

sensitivity of this PCR technique no additional normal or malignant breast tissue will be removed.

Risks

Subject: None above the standard risks involved with standard blood draw. Blood drawing risks include bruising pain swelling and discomfort. A pressure bandage will be applied to the spot of the blood draw to minimize the swelling.

Investigators: Handling of blood products and human tissue.

Benefits

None

Funding

The evaluation of blood tests is funded by the Department of Defense Breast Cancer Research Program.

Special Consent issues

The research protocol and the purpose of the study will be explained to each study participant. The consent will be obtained by the primary investigator, the co-investigators, or a clinical nurse in the cancer center on behalf of the investigators. Informed consent will be obtained in the clinic not more than two weeks prior to surgery. Blood will be drawn after the informed consent is signed. Blood drawing will occur in the clinic.

Modification of the Protocol

Any modifications made to the protocols and/or consent forms must be submitted to the local IRB and to the Human Subjects Research Review Board (HSRRB) for review and approval before implementation.

Roles and Responsibilities of Study Personnel

Dr. Sclafani will be responsible for supervising laboratory personnel. Laboratory study personnel will be responsible for storing and evaluating samples. They will carry out the assays as described above and evaluate data that is obtained. Dr. Kraft will take responsibility for protocol entry and sample collection.

Adverse Events

Adverse experiences that are both serious and unexpected will be immediately reported by telephone to the USAMRMC Deputy for Regulatory Compliance and Quality (301-619-2165) (non-duty hours call 301-619-2165 and send information by facsimile to 301-619-7803). A written report will follow the initial telephone call within 3 working days. Address the written report to the US Army Medical Research and Materiel Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

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Consent Form Approval

Allan Prochazka, MD/Stephen Bartlett, R.Ph., Co-Chairs, COMIRB
Christopher Kuni, MD/Ken Easterday, R.Ph., Co-Chairs, COMIRB
Adam Rosenberg, MD/David Lawellin, Ph.D., Co-Chairs, COMIRB

Date: _____ Valid Through: _____

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD

"Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay"

**Principal Investigator: Andrew S. Kraft
SUBJECT CONSENT FORM
April 11, 2000/COMIRB Protocol Number 00-848**

Project Description

You are being asked to take part in an investigational study evaluating the use of a blood assay to measure the levels of a specific modified piece of DNA in your blood. Breast cancer is caused in part by the lack of the ability of cells to produce specific proteins that inhibit the growth of cells. This lack of protein production is caused by modification of cellular DNA. This DNA abnormality is not an inherited trait and the reason it occurs is not known. Since tumor cells are growing and dying some of this abnormal DNA may be released into the blood. Using a newly developed laboratory assay, we would like to attempt to measure this abnormal DNA in your blood. Sixty women with metastatic breast cancer will be enrolled into this study. Participation in this study is voluntary and the purpose of this consent is to inform you about the study and its possible benefits and risks.

Procedures

If you agree to participate, you will need to donate a single 30ml of blood (two tablespoons) at a time when you are known to have breast cancer. This blood will be sent to the lab for evaluation of abnormal DNA levels.

Initials _____

Discomforts and Risks

Venipuncture Risk

Approximately 2 tablespoon of blood will be removed by putting a needle into your vein at the time of your pre-operative evaluation. This is the standard method used to obtain blood for tests. You will feel pain when the needle goes into the vein. A bruise may form at the site. A total of 30 ml will be taken for research purposes over the course of this study.

Benefits

You will receive no benefit from participating in this research study and there are risks as mentioned in the risk section.

Source of Funding

All funding for this study will be provided by the Department of the Army's Breast Cancer Research Program.

Cost to Subject

There is no cost to you for participating in this study. There will be no charge for procedures or labs required by the study. You will not be paid for your participating in this study.

Study Withdrawal

You may choose not to enter the study or withdraw from the study at any time and your doctor will continue to take care of you without loss of benefits to which you are entitled. Your doctor may also choose to withdraw you from the study at any time if he/she feels that it would be harmful to your health for you to continue or the side effects are too severe. Significant new findings that relate to your participation in this study will be discussed with you.

Invitation for Questions

You will receive a copy of this consent form. Please ask questions about this research or consent either now or in the future. You may direct your questions to Dr. Andrew S. Kraft, MD 303-315-8802 or Dr. Robert Sciafani at (303) 315-7288. If you have questions regarding your rights as a research subject, please call the Colorado Multiple Institutional Review Board (COMIRB) office at (303) 315-7960.

Confidentiality

Your physician/investigator will treat your identity with professional standards of confidentiality. However, the U.S. Department of Health and Human Services, and the Colorado Multiple Institute Review Board have the right to inspect all of your medical records relating to this research for the purpose for verifying data. It should be noted that representatives of the US Army Medical Research and Material Command are eligible to review research records as a part of their responsibility to protect human subjects in research. The principal investigator or a designee will review your chart in order to identify information regarding the stage of your breast cancer. The information

Initials _____

obtained in this study may be published in medical journals, but your identity will not be revealed.

A code will be assigned to your sample and the data it generates that can be assessed only by the principal investigators of this study. The code will be kept in a locked cabinet. The samples will be stored for 10 years.

Injury and Compensation

If you are hurt by this research, we will provide medical care if you want it. The United States Department of Defense is funding this research project. Should you be injured as a direct result of participating in this research project, you will be provided medical care at no cost to you, for that injury. You will not receive any injury compensation, only medical care. You should also understand that this is not a waiver or release of your legal rights. You should discuss this issue thoroughly with the principal investigator before you enroll in this study. You will not be paid for any other loss as a result of the injury, such as loss of wages, pain and suffering. Further information can be obtained by calling Andrew S. Kraft, MD 303-315-8802.

AUTHORIZATION:

I have read this paper about the study or it was read to me. I know what will happen, both the possible good and bad (benefits and risks). I choose (or allow my child) to be in this study. I know I can stop being in this study and I (or my child) will still get the usual medical care. I will get a copy of this consent form. (Initial all the previous pages of this consent form)

Signature: _____ Print Name _____ Date _____
Subject, parent or guardian

Permanent Address of Subject, parent or guardian

Consent form explained by: _____ Print Name _____ Date _____

Investigator _____ Date _____

Initials _____

Consent Form Approval

Allan Prochazka, MD/Stephen Bartlett, R.Ph., Co-Chairs, COMIRB
Christopher Kuni, MD/Ken Easterday, R.Ph., Co-Chairs, COMIRB
Adam Rosenberg, MD/David Lawellin, Ph.D., Co-Chairs, COMIRB

Date: _____ Valid Through: _____

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD

"Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients"

Principal Investigator: Andrew S. Kraft
SUBJECT CONSENT FORM
April 11, 2000/COMIRB Protocol Number 00-849

Project Description

You are being asked to take part in an investigational study evaluating the use of a blood assay to measure the levels of a specific modified piece of DNA in your blood. Breast cancer is caused in part by the lack of the ability of cells to produce specific proteins that inhibit the growth of cells. This lack of protein production is caused by modification of cellular DNA. This DNA abnormality is not an inherited trait and the reason it occurs is not known. Since tumor cells are growing and dying some of this abnormal DNA may be released into the blood. Sixty women will be enrolled into this study with 20 women each having Stage II, III or IV breast carcinoma. This study will identify whether the abnormal DNA found in your breast cancer is also found in your blood. Participating in this study does not mean that you have any hereditary abnormality in your DNA. This study will measure the levels of abnormal DNA found in your tumor and attempt to correlate the results of this blood test with the tumor sample. Participation in this study is voluntary and the purpose of this consent is to inform you about the study and its possible benefits and risks.

Procedures

If you agree to participate, you will need to donate 30ml of blood (two tablespoons), at the time of your pre-operative evaluation. This blood will be sent to the lab for evaluation of free DNA. During your operation, one gram of normal breast tissue (about the size of a pea) will be removed from your breast (which is undergoing surgery). This small sample of normal breast tissue, as well as one gram of the breast tumor, will be taken to a research lab for processing and further study after it has been viewed by the pathologist. The findings in the research lab will then be correlated with the results of

Initials _____

the blood test. No additional tumor or breast tissue will be removed for this study other than that required during your operation. The study will be completed on the material not needed for pathologic examination. You will not receive any information regarding these additional tests.

Discomforts and Risks

Breast Biopsy

At the time of surgery, after the cancer has been removed, one gram of normal breast tissue (about the size of a pea) will be removed. If you are having a lumpectomy, after the cancer is removed, one gram of normal breast tissue (about the size of a pea) will also be removed for the study. If you are having a mastectomy, after the breast is removed, one gram of normal tissue will be taken from the breast for the study. There will be no additional risk to you other than the usual risks for your surgery.

Venipuncture Risk

Approximately 2 tablespoon of blood will be removed by putting a needle into your vein at the time of your pre-operative evaluation. This is the standard method used to obtain blood for tests. You will feel pain when the needle goes into the vein. A bruise may form at the site. A total of (30 ml) will be taken for research purposes over the course of this study.

Benefits

You will receive no benefit from participating in this research study and there are risks as mentioned in the risk section.

Source of Funding

All funding for this study will be provided by the Department of the Army's Breast Cancer Research Program.

Cost to Subject

There is no cost to you for participating in this study. There will be no charge for procedures or labs required by the study. You will not be paid for your participating in this study.

Study Withdrawal

You may choose not to enter the study or withdraw from the study at any time and your doctor will continue to take care of you without loss of benefits to which you are entitled. Your doctor may also choose to withdraw you from the study at any time if he/she feels that it would be harmful to your health for you to continue or the side effects are too severe. Significant new findings that relate to your participation in this study will be discussed with you.

Invitation for Questions

You will receive a copy of this consent form. Please ask questions about this research or consent either now or in the future. You may direct your questions to Dr. Kraft at (303) 315-8802 or Dr. Robert Sclafani at (303) 315- 7288 If you have questions

Initials _____

regarding your rights as a research subject, please call the Colorado Multiple Institutional Review Board (COMIRB) office at (303) 315-7960.

Confidentiality

Your physician/investigator will treat your identity with professional standards of confidentiality. However, the U.S. Department of Health and Human Services, and the Colorado Multiple Institute Review Board have the right to inspect all of your medical records relating to this research for the purpose for verifying data. It should be noted that representatives of the US Army Medical Research and Material Command are eligible to review research records as a part of their responsibility to protect human subjects in research. The principal investigator or a designee will review your chart in order to identify information regarding the stage of your breast cancer. The information obtained in this study may be published in medical journals, but your identity will not be revealed.

A code will be assigned to your sample and the data it generates that can be assessed only by the principal investigators of this study. The code will be kept in a locked cabinet. The samples will be stored for 10 years.

Injury and Compensation

If you are hurt by this research, we will provide medical care if you want it. The United States Department of Defense is funding this research project. Should you be injured as a direct result of participating in this research project, you will be provided medical care at no cost to you, for that injury. You will not receive any injury compensation, only medical care. You should also understand that this is not a waiver or release of your legal rights. You should discuss this issue thoroughly with the principal investigator before you enroll in this study. You will not be paid for any other loss as a result of the injury, such as loss of wages, pain and suffering. Further information can be obtained by calling Andrew S. Kraft, MD 303-315-8802.

AUTHORIZATION:

I have read this paper about the study or it was read to me. I know what will happen, both the possible good and bad (benefits and risks). I choose (or allow my child) to be in this study. I know I can stop being in this study and I (or my child) will still get the usual medical care. I will get a copy of this consent form. (Initial all the previous pages of this consent form)

Signature: _____ Print Name _____ Date _____
Subject, parent or guardian

Permanent Address of Subject, parent or guardian

Initials _____

Consent form explained by: _____ Print Name _____ Date _____

Investigator _____ Date _____

/ / / /

Initials _____

Protocol Summary

Project Title: Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay.

Investigators: Andrew S. Kraft, MD, Christina A. Finlayson, MD, Robert Sclafani, Ph.D. and Thomas A. Langan, Ph.D.

COMIRB# 00-848

Date: August 28th, 2000

Revised October 6th, 2000

Background/Rationale

The importance of defects in the Rb pathway in the development of cancer is indicated by the fact that almost all cancer cells are defective in some aspect of its regulation (1-5). This near universal prevalence of defects in components of the Rb pathway suggests that overcoming normal cell cycle regulation at this point is necessary condition for the development of malignancy (5). These defects include loss of expression of Rb (6,7), overexpression of cyclin D1 (8), and loss of expression of p16 inhibitor protein, whose role as a tumor suppressor is now well documented (9). In particular, the importance of p16 in tumor suppression is seen in that homozygous p16 deletions in mice result in spontaneous development of multiple tumor types (10).

Recognition of the importance of p16 as a tumor suppressor comes in part from the recent discovery that gene methylation, in addition to homozygous deletion or loss of heterozygosity seen for other tumor suppressor genes is a major mechanism for inactivation of p16 gene in all common human cancers (11,12). In primary breast tumors, which only rarely show homozygous p16 deletions or point mutations (13,14), 31% of tumors were found to contain p16 genes inactivated by methylation (11). We hypothesize that p16 gene methylation is responsible for uncontrolled cellular proliferation in many breast tumors, and that agents that increase this protein will prevent the growth of cancer. For example, DHAC, an NCI-produced (NSC #264880) methylation inhibitor has been tested in phase I and phase II clinical trials.

It has been known for several years that the plasma component of circulating blood contains tiny quantities of free DNA. The concentration of DNA in the plasma of healthy individuals is approximately 14 ng/ml DNA'. This level increases significantly in individuals diagnosed with different types of cancer to approximately 180 ng/ml².

The ability to detect tumor-specific molecular defects in the circulating blood would obviate the requirement for tumor biopsy material thus providing a more efficient and noninvasive means of screening for a vast array of molecular aberrations. Our objective is to identify women whose tumors contain methylated p16 genes for recruitment into a clinical trial that aims to test the efficacy of therapeutic demethylating

agents. We therefore propose to determine the feasibility of assaying plasma DNA for the presence of methylated p16 alleles in women diagnosed with metastatic breast cancer. DNA will be extracted from circulating blood plasma then assayed for p16 promoter methylation status by methylation-specific PCR (MSP)³. The normal breast tissue is expected only to contain unmethylated p16 whereas the tumor tissue may contain either unmethylated or methylated p16. If the tumor DNA contains methylated p16 alleles, we should also expect to see methylated p16 in the plasma DNA in addition to normal cell-specific unmethylated p16.

Hypothesis

We hypothesize that a PCR based assay method can be used to detect methylated p16 gene in the blood of breast cancer patients. This test will replace the necessity of obtaining breast cancer tissue to measure the methylation of this gene.

Purpose

The data from this study will determine the utility of this PCR based blood test in detecting methylated or unmethylated p16 DNA in the blood of breast cancer patients. Since 30% of breast cancer patients are expected to have methylated p16 DNA in their tumors, the data collected in this study will begin to evaluate the sensitivity of this blood test.

Methods

Women being evaluated in breast cancer clinic who are found to have metastatic disease will be asked to sign a consent allowing us to obtain a blood sample (20 ml). All data concerning the extent of disease and disease progression will be kept confidential. The patient will be identified by number only and the data will be kept in locked files.

- Isolation of DNA from blood plasma

Using 10 ml plasma (from approximately 20 ml whole blood collected in EDTA containing tubes), we have optimized the following isolation procedure to yield sufficient DNA for an initial MSP assay plus three further confirmatory reactions.

The 10 ml plasma sample (which may be frozen at -80°C prior to analysis) is first heated at 99°C for 5 minutes then centrifuged at high speed to allow recovery of the clear, DNA containing, supernatant. Following an overnight incubation with proteinase K the DNA is purified on QIAamp columns (QIamp Blood Kit; Qiagen Inc., Hilden, Germany) according to the Blood and Body Fluids protocol.

- **Methylation-specific PCR analysis of plasma**

The total plasma DNA sample is then used for the MSP assay. The DNA is first modified overnight with sodium bisulfite which converts only unmethylated cytosines to uracil. Following purification on Wizard columns (Promega, Inc.), the DNA is precipitated with ammonium acetate and ethanol. One-tenth of the resulting DNA is then subjected to PCR analysis using oligonucleotides designed from the promoter of the p16 gene that are specific for wild type (unmodified), methylated and unmethylated DNA sequences.

We have performed MSP analysis of multiple independently-isolated normal plasma DNA preparations and, as expected, shown amplification using the unmethylated DNA-specific oligonucleotides only. Furthermore, we have titrated cell line DNA containing methylated p16 alleles into plasma samples prior to isolation of plasma DNA and shown that we can successfully amplify as little as 12.5 ng methylated DNA in addition to the unmethylated normal plasma DNA using this method. Because the normal plasma DNA contains only unmethylated DNA, this system will enable us to identify tumor-specific methylated DNA in the plasma of breast cancer patients.

Observations

- 1- We would plan to determine that the above assay could be done on actual patient material.
- 2- Since approximately 30% of the tumors are positive to p16 methylation, we would plan to test whether the assay picks up this number of tumors.
- 3- We would plan to correlate the stage and extent of disease with the number of positive assays.

Inclusion criteria

Woman ages 18-72 years that are known to have metastatic breast cancer as determined by x-ray and clinical history.

Exclusion criteria

- 1- Patients deemed not competent to make their own decision
- 2- Patients with a previous history of another cancer.

Sample Size

The sample size will be 100 patients. We will be able to establish a confidence interval of at least ± 0.1 on the blood samples with a sample size of 100. This can be established regardless of the sensitivity or specificity of the assay in either case. We will be able to provide a similar confidence interval for the proportion detected by the blood test and see if that covers the anticipated 30% prevalence of methylation.

Estimated Duration of Study

The estimated duration of the study is 1 year. If the samples are collected more quickly the study will be terminated.

Examinations and evaluations

Other than the drawing of 20 cc of blood no further examination of the patient will be done. The extent of metastatic breast cancer will be noted from the chart.

Drugs, devices or instruments

No novel drugs devices or instruments are involved in this study.

Data analysis

The data analysis will be carried out by Dr. J. Murphy in biostatistics.

Data Security

All electronic data will be secured in protected files. All paperwork will be secured under lock and key. The data will be coded and the master file placed in a locked file cabinet in the Principal Investigators office.

Changes from Usual Treatment

There will be no changes in treatment. All participants will receive therapy deemed as standard of care by their primary oncologist.

Risks

Subject: None above the standard risks involved with standard blood draw.

Investigators: Handling of blood products.

Benefits

None

Funding

The evaluation of blood tests is funded by the Department of Defense Breast Cancer Research Program.

Special Consent issues

The research protocol and the purpose of the study will be explained to each study participant. The consent will be obtained by the primary investigaotr, the co-investigators, or a clinical nurse in the cancer center on behalf of the investigators. All of the investigators getting consent will either have COMIRB training or been trained by a Principal investigator who has completed COMIRB training.

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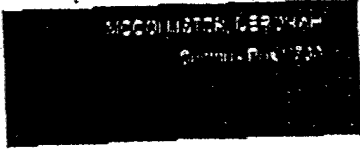


Colorado Multiple Institutional Review Board
 13001 E. 17th Place
 Building 500, Room N3214
 Aurora, Colorado 80010-7238

Mailing Address:
 Mail Stop F-490
 P.O. Box 6508
 Aurora, Colorado 80045-6508

(303) 724-1055 [Phone]
 (303) 724-0990 [Fax]
 comirbweb.uchsc.edu [Web]
 comirb@uchsc.edu [E-Mail]
 Assurance # M-1494-01

University of Colorado Hospital
 Denver Health Medical Center
 Veterans Administration Medical Center
 The Children's Hospital
 University of Colorado Health Sciences Center
 Colorado Prevention Center



02/12/2002

Certificate of Approval

Investigator: **ANDREW KRAFT**
 Sponsor: **ARMY CTR AWARD**
 Subject: **COMIRB Protocol 00-849
 Continuing Review (CRV001)
 1st Review**

Title:
**CORRELATION BETWEEN METHYLATED P16 DNA IN THE BLOOD AND
 BREAST CANCER TISSUE OF PATIENTS**

Approval Date: **9 October 2001**
 Expiration Date: **9 October 2002**

Approval Includes: **Protocol - Investigator - Consent and/or Assent Form - Continuing Review**

- All COMIRB Approved Investigators must comply with the following:
- For the duration of your protocol, any change in the experimental design/consent and/or assent form must be approved by the comirb before implementation of the changes.
 - Use only a copy of the COMIRB signed and dated Consent and/or Assent Form. The Investigator bears the responsibility for obtaining from all subjects "Informed Consent" as approved by the COMIRB. The COMIRB REQUIRES that the subject be given a copy of the consent and/or assent form. Consent and/or assent forms must include the name and telephone number of the investigator.
 - Provide non-English speaking subjects with a certified translation of the approved Consent and/or Assent Form in the subject's first language. A copy of the translator's certification should be attached to the consent and/or assent form.
 - The investigator also bears the responsibility for informing the COMIRB immediately of any Serious Adverse Events (deaths, serious complications or other untoward effects of this research at this or other sites), and of the relationship of the SAE to the investigational trial. The COMIRB uses the standard definition of Serious or Unanticipated Events that include: death, hospitalization, prolongation of hospitalization and other unanticipated side effects
 - Obtain COMIRB approval for all advertisements before use.
 - Federal regulations require a Continuing Review to renew approval of this project within a 12-month period from the last approval date unless otherwise indicated in the review cycle listed below. If you have a restricted/high risk protocol, specific details will be outlined in this letter. Non-compliance with Continuing Review will result in the termination of this study. This project has been assigned the following review cycle:

COMIRB Continuing Review Cycle: 12 months

We will send you a Continuing Review Form to be completed prior to the due date. Any questions regarding the COMIRB action on this study should be referred to the COMIRB staff at 303-724-1055 or UCHSC Box F-490.

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 Ken Easterday, RPh Co-Chair

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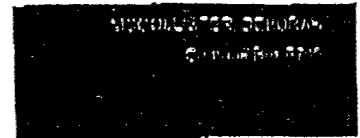


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 Mail Stop F-490
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(303) 724-1055 [Phone]
 (303) 724-0990 [Fax]
 comirb@wob.uchsc.edu [Web]
 comirb@uchsc.edu [E-Mail]
 Assurance # M-1494-01

University of Colorado Hospital
 Denver Health Medical Center
 Veteran's Administration Medical Center
 The Children's Hospital
 University of Colorado Health Sciences Center
 Colorado Prevention Center



02/12/2002

Certificate of Approval

Investigator: **ANDREW KRAFT**
 Sponsor: **ARMY CTR AWARD**
 Subject: **COMIRB Protocol 00-848
 Continuing Review (CRV001)
 2nd Review**

Title:
**ESTIMATION OF ABNORMAL BREAST CANCER DNA IN PATIENT'S BLOOD
 USING A SENSITIVE PCR BASED ASSAY**

Approval Date: **23 October 2001**

Expiration Date: **23 October 2002**

Approval Includes: **Protocol - Investigator - Consent and/or Assent Form - Continuing Review**

All COMIRB Approved Investigators must comply with the following:

- For the duration of your protocol, any change in the experimental design/consent and/or assent form must be approved by the comirb before implementation of the changes.
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Revised 01/02