Influence of Perceived Discrimination and Stress on Tumor Biology and Disease Outcome through Activation of the Biobehavioral Catecholamine Pathway in Human Breast Tumors

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PRECIS

We are proposing a biobehavioral and clinical research study that will evaluate the relationship of perceived stress, social isolation, and discrimination among female breast cancer patients with prognostic tumor markers. We will recruit approximately 100 consented patients, but not more than 150 patients, who will have a surgical resection of their cancerous breast at either the University of Maryland Medical Center or the Baltimore Washington Medical Center. Patients must have a pathologically confirmed diagnosis of breast cancer and must be able to speak English, give informed consent, be physically and mentally capable of performing the interview, and cannot reside in an institution, such as a prison, nursing home, or shelter. We will obtain a non-objection statement to contact the patient by the treating physician from the hospital where the patient was identified (Drs. Kesmodel, Bellavance, Drogula). Patients will be mostly African-American and European-American patients, representing the patient pool in the greater Baltimore area and the two hospitals. Recruitment is planned for a duration of 15 to 24 months, starting in 2012. We will collect fresh-frozen cancerous and adjacent non-cancerous tissue from routine surgery and blood samples (for preparation of plasma/serum, peripheral blood monocytes) from these patients. The patients will also complete an interviewer-administered survey before they have surgery that evaluates perceived stress and discrimination within the last month, perceived social isolation, use of beta blockers, education and income, marital status, self-reported race/ethnicity, and body mass index. The study will not perform any procedures on these patients that are not part of standard care with the exception of completing the short interviewer-administered questionnaire, obtaining blood samples by a trained phlebotomist, and obtaining tumor tissue and adjacent normal tissue after surgical resection. The proposed research study is testing the hypothesis that perceived stress, and particularly discrimination and social isolation-induced stress, influence tumor biology and are associated with a prognostic gene expression signature and increased tumor catecholamine levels in breast tumors, linking stress exposure to the activation of the pro-metastatic catecholamine pathway and a poor outcome phenotype in breast cancer.

1. BACKGROUND

Cancer epidemiology has linked racial discrimination to breast cancer development and stressful life events to poor disease survival (1,2). Chronic stress influences tumor biology through two major pathways involving catecholamines and glucocorticoids (3,4). Stress-induced beta-adrenergic signaling has been shown to promote cancer progression and metastasis in animal models of breast and ovarian cancer (5-7). Others showed that catecholamines have pro-metastatic properties in cancer, which can be blocked by inhibitors of the beta-adrenergic signaling pathway, such as beta-blockers like propranolol (8,9). Recently, social isolation was found to be associated with an elevated tumor catecholamine level in human ovarian tumors and a distinct tumor gene signature, albeit the latter study was rather small-sized including only 20 ovarian cancer patients (10,11). More recently, it was found that beta-blocker use among patients reduces disease recurrence and improves cancer-specific survival of breast cancer patients (12). Propranolol users are significantly less likely to develop advanced breast cancer and have a reduced breast cancer-specific mortality (13). Moreover, beta-blocker use has been associated with improved recurrence-free survival in the triple-negative disease (14). Jointly, these data indicate that stress may influence human breast cancer biology through activation of the pro-metastatic catecholamine pathway, leading to a poor outcome phenotype in a subpopulation of patients who would benefit from beta-blocker use and stress management.

2. STUDY OBJECTIVES

We are pursuing the hypothesis that stress, social isolation, and discrimination are associated with a prognostic gene expression signature and with increased tumor adrenaline and noradrenaline levels in breast cancer. We also hypothesize that the stress-induced signature is more common in breast tumors of African-American (AA) than European-American (EA) patients and is a biomarker for catecholamine signaling and can be used to 1. Select patients for intervention therapy (e.g., beta-blocker, stress management) and 2. Identify novel blood-based markers of stress-related tumor progression.

Specific Aims

1. Examine relationship between self-reported perceived stress/discrimination and tumor biology by analyzing tissue gene expression profiles and selected protein markers

2. Examine relationship between perceived stress/discrimination, tumor characteristics, and tumor catecholamine levels

3. Generate tumor gene signature for catecholamines and compare this signature with stress/discrimination signature

4. Evaluate gene signature(s) as predictor of disease outcome and response to therapy using in-house and publically available datasets for breast cancer

5. Evaluate prevalence of stress signature in breast tumors from AA and EA patients and by tumor subtype.

6. Identify candidate blood-based marker(s) for stress/discrimination/catecholamine-induced tumor progression using the obtained gene expression profiles for guidance. As our future course, evaluate these marker(s) in the current study and in a cohort of AA and EA breast cancer patients to be recruited at Georgetown University by Vanessa Sheppard and colleagues.

3. RATIONALE

Rationale to Study Discrimination- and Stress-induced Molecular Profiles In Breast Tumors and Blood Samples

Recent progress in whole genome expression profiling has generated prognosis-related molecular profiles for many cancer types. Gene expression profiling has been very successful in breast cancer research (15,16) and identified gene signatures, e.g., the 70-Gene MammaPrint and Oncotype DX signatures, which are now used to guide clinical treatment decisions (17,18). Thus, this technology allows us to identify subsets of patients who have a specific tumor biology that influences disease prognosis, either because of intrinsic tumor factors or because of an environmental exposure that shapes tumor biology. We believe that gene expression profiling is a very appropriate tool to describe the possible influence of stress-related exposures on breast cancer biology and pilot study of ovarian cancer has shown that stress-related prognostic tumor signature may well exist (10). In addition, we will use marker analysis, such as the direct

measurements of tumor adrenaline and noradrenaline levels, to validate self-reported stress exposures, as previous research has shown that stress influences tumor biology through the pro-metastatic catecholamine pathway involving adrenaline and noradrenaline and cAMP signaling (4). Together, these markers (gene expression profiles and catecholamine levels) should be most appropriate in evaluating discrimination/stress-based effects in breast cancer biology. Moreover, a very large and publically available archive of gene expression data for more than 1500 human breast tumors exits that can be used to evaluate the prognostic significance of any newly discovered signature, e.g., a stress-related signature. There is no other technology than gene expression profiling that would give one the opportunity to relatively quickly determine the prevalence of a tumor biology-related signature in breast cancer across large datasets, and how this signature is related to tumor subtypes, response to therapy, and disease outcome. Lastly, we will use the obtained gene expression profiling data to select candidate blood-based markers of stress-induced tumor progression. Currently, there are no such markers existing that could tell us whether a breast cancer patient would benefit from a beta blocker intervention therapy or stress management for better survival. We hypothesize that pro-metastatic catecholamine signaling in breast tumors generates a distinct protein and/or metabolite-based profile in blood, or a distinct methylation profile in peripheral blood monocytes, which can be used for patient selection. Existing data showing that stress exposures can be linked to leukocyte telomere length support this hypothesis (19,20).

Rationale for Conducting the Study in the Greater Baltimore Area and recruiting patients at the University Maryland Medical Center and the Baltimore Washington Medical Center

Baltimore City and the patients at the two hospitals come from a very diverse community with many residents being part of the American middle class with a working class majority. However, Baltimore has many poverty stricken neighborhoods with mostly AA residents, who experience discrimination on a daily basis. The cultural, race/ethnic, and sociodemographic diversity of the city and of the patients coming to the two hospitals make the area and the institutions an ideal study location to evaluate the influence of discrimination and stress on breast cancer biology, as proposed by our study. It is one of our objectives to estimate the prevalence of a tumor stress signature in AA and EA

patients because breast cancer has a large survival health disparity between the two patient groups, with AA patients experiencing an excess mortality. The causes of this survival health disparity are multifactorial, but it has been proposed that perceived discrimination and stress have a more detrimental impact on health in the AA patient group, leading to a poor outcome cancer phenotype in a subset of patients and a higher mortality among these patients. Thus, performing the study at the two hospitals would allow us to the study this difference between AA and EA patients because these hospitals treat about equal numbers of AA and EA breast cancer patients. In addition, the surgeons at the University Maryland Medical Center and the Baltimore Washington Medical Center have performed in excess of 100 mastectomies per year in past years. Thus, the proposed collection of about 100 surgically resected tumor specimens is realistic and can be achieved in a 12 to 15 months period.

4. ELIGIBILITY ASSESSMENT AND ENROLLMENT

Patients will be identified through contacts with three physicians: Drs. Susan Kesmodel, Emily Bellavance, and Cynthia Drogula. We will contact only patients that are scheduled to have surgery. Patient recruitment will follow HIPAA regulations ("HIPAA Privacy Rule"). After the eligibility has been confirmed, a designated study interviewer and the treating physician will meet with the patients to introduce the study and get informed consent, authorization to obtain tissue, and authorization to use and disclose protected health information for research from those who want to participate. After informed consent has been obtained, a blood sample will be collected and the survey will be administered by the designated study interviewer. A second blood sample will be obtained after surgery (or approximately 4 weeks after the first blood sample has been obtained). Alternatively, the patients may be visited at their home for the interview and blood collection.

Procedures

1. Treating physician identifies and the interviewer or physician screens the patient prior to the patient's surgery.
2. The patient's name, location, physician's name, and age are recorded. If the patient is not eligible, reasons for ineligibility are recorded.
3. The treating physician and an interviewer meet the patients at their appointment and obtain informed consent, authorization to obtain tissue, and

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authorization to use and disclose protected health information for research. Collect blood in two 7 cc red top tubes and four 10 cc green top tubes. Place tubes in a thermos for short-term storage and transport.

4. Provide incentive of $25.
5. Perform interview and complete questionnaire for those consented (always prior to surgery). The interviewer will give a copy of the questionnaire to the patient before the interview begins. The patient will have the opportunity to read a question while being interviewed. Collect blood in two 7 cc red top tubes and four 10 cc green top tubes. Place tubes in a thermos for short-term storage and transport.
6. Provide incentive of $25.
7. Blood (separation of serum and blood clot; buffy coat; plasma and red blood cells) to be processed within 8 hours at the University of Maryland Department of Pathology. Freeze about 20 million PBMCs in 5 million per vial aliquots.
8. At day of surgery, surgical specimens will be obtained (tumor and adjacent nontumor tissue) and will be processed within 20 to 30 minutes after removal. A portion will be placed in OCT and another snap frozen in the vapor phase of liquid nitrogen.
9. Review pathology report to confirm diagnosis and abstract pathological data. Also record histology of tumor.
10. Collect a second blood sample after surgery (preferentially at the next follow up visit for the patients) in two 7 cc red top tubes and four 10 cc green top tubes. Place tubes in a thermos for short-term storage and transport.
11. Blood (separation of serum and blood clot; buffy coat; plasma and red blood cells) to be processed within 8 hours at the University of Maryland Department of Pathology. Freeze about 20 million PBMCs in 5 million per vial aliquots.
12. If subject is re-contacted at a later time for additional phlebotomy, then an additional $25 incentive is given. If subject is contacted for additional questionnaire information, then no additional award is given

Re-contacting subjects for additional information or blood

The interviewer who administered the questionnaire will be the person who will re-contact the subject. This re-contact will not involve a script because of the large number of possible circumstances. Subjects will be re-contacted only under certain conditions. The conditions are as follows:

1. If there is missing information in the questionnaire
2. If there is a discrepancy between questionnaire and medical record information
3. If an insufficient volume of blood has been collected
4. If there has been mishandling of a blood sample in the laboratory
5. If there has been an accident leading to the loss of a blood sample
6. If the analysis of a blood sample returns a highly unusual result

We will not contact subjects, who refused to answer questions during the interview process, for responses to those questions they refused. Missing information due to refusal will be marked as such in the questionnaire. We will also not contact a person who failed to complete the sexual history part of the questionnaire.

4.1. ELIGIBILITY CRITERIA

Selection of patients

We will recruit incident female patients of pathologically confirmed breast cancer at all stages of the disease that are age 30 to 90 years. Only patients with scheduled surgery are eligible. The following check list will be used to verify eligibility of a patient subject.

ELIGIBILITY CHECK LIST

NAME_____   DATE_____

ID# _____

Yes	No	Criteria (ALL MUST BE CHECKED)
		Pathological diagnosis of breast cancer made at the local hospital pathology department
		Being a female patient
		Age 30 to 90 years
		A non-objection statement by the physician from the hospital where the patient was identified (Drs. Susan Kesmodel, Emily Bellavance, and Cynthia Drogula)
		Is not currently residing in an institution, such as a prison, nursing home, or shelter
		Is not a severely ill patient in the intensive care unit
		Speaks English well enough to be interviewed
		Is able to give informed consent
		Is physically and mentally capable of performing the interview
		Subject provides informed consent and signs form. _____Unwilling _____Unavailable

Inclusion criteria

1. Pathological diagnosis of breast cancer made
2. Being a female patient
3. Age 30 to 90 years
4. Speaks English well enough to be interviewed
5. Is physically and mentally capable of performing the interview

6. A non-objection statement by the physician from the hospital where the patient was identified (Drs. Susan Kesmodel, Emily Bellavance, and Cynthia Drogula)

Exclusion criteria

1. Severely ill subjects in the intensive care unit (they can be reconsidered after discharge from ICU)
2. Currently residing in an institution, such as a prison, nursing home, or shelter
3. Patient is unable to give informed consent

5. STUDY IMPLEMENTATION

The study will be implemented when IRB approval at the University of Maryland has been obtained. The study is scheduled for a time period of 15-24 months, or when a maximum of 150 patients have been recruited. The current protocol will take place under the Office of Budget and Management-reviewed and approved multi-organ contract to recruit cancer patients with an epidemiological profile, which is in place for more than 20 years, a protocol titled "Resource for the collection and evaluation of human tissues and cells from donors with an epidemiology profile", University of Maryland at Baltimore IRB #0298229.

Study Personnel

The Principal Investigator, Dr. Stefan Ambs, will be responsible for the overall monitoring of the study. The collaborating physicians at the University of Maryland, Drs. Susan Kesmodel, Emily Bellavance, and Cynthia Drogula, will support the study by informing their patients that this study exists, and by helping our study coordinators to contact/meet the patients if deemed eligible for the study. The physicians will also assist in securing the surgically resected breast tissue by study personnel after surgery. The study coordinator and contractor is the University of Maryland Pathology Department. An existing NCI contract supports the established epidemiological infrastructure maintained by the University of Maryland Pathology Department contractor. Personnel include a PI for the contract (Dr. Mann) and a field supervisor (Ms. Perlmutter), and trained interviewers and phlebotomist, and staff to collect and process biological

specimens, data entry personnel, and a statistician and epidemiologist for data analysis and quality control assessment. Leoni Leondaridis, a software consultant to the Laboratory of Human Carcinogenesis, will assist with database development and management.

Previous experience of the University of Maryland Pathology Department contractor with patient recruitment

The contractor has extensive experience in recruiting cancer patients at the University of Maryland Medical Center and other hospitals in the Greater Baltimore area. The contractor recruited about 400 breast cancer patients between 1993 and 2004. The contractor is currently recruiting lung, prostate, liver and pancreatic cancer patients for NCI studies. Many of the procedures described for this protocol are identical with procedures that were established to recruit patients with other cancer types into existing protocols. The current protocol will take place under the Office of Budget and Management-reviewed and approved multi-organ contract to recruit cancer patients with an epidemiological profile, which is in place for more than 20 years, a protocol titled "Resource for the collection and evaluation of human tissues and cells from donors with an epidemiology profile", University of Maryland at Baltimore IRB #0298229.

6. STUDY DESIGN

We will recruit approximately 100 consented patients, but not more than 150 patients, who will have a surgical resection of their cancerous breast at either the University of Maryland Medical Center or the Baltimore Washington Medical Center. Patients must have a pathologically confirmed diagnosis of breast cancer and must be able to speak English, give informed consent, physically and mentally capable of performing the interview, and cannot reside in an institution, such as a prison, nursing home, or shelter. We will collect blood before and after surgery and tissue (tumor and adjacent non-cancerous tissue) at time of surgery.

Questionnaire

Trained interviewers will administer the questionnaire prior to surgery. Only at the rare occasion that an interview cannot be completed prior to surgery, the questionnaire may be administered at the next follow up visit after surgery. The interview will last approximately 30 to 40 minutes. The questionnaire evaluates perceived stress within the last month, chronic discrimination exposure and currently perceived social isolation, use of beta blockers, education and income, marital status, self-reported race/ethnicity, and body mass index.

Interviewer training

The interviewers will receive a procedure manual. They will be trained in how to identify eligible subjects, how to provide informed consent, how to administer and properly complete the questionnaire, how to perform phlebotomy, and how to properly process blood samples. The field supervisor will provide the training. Newly hired interviewers will practice the administration of the questionnaire to office volunteers. Interviewers will then administer the questionnaire and draw blood from subjects under the supervision of the field supervisor, who will provide feedback after the interview.

7. COLLECTION AND PROCESSING OF BIOLOGICAL SAMPLES

We will collect tumor specimens and blood samples. One blood sample will be collected prior to surgery and a second one after surgery. The collected blood will be used for the analysis of markers associated with a discrimination/stress signature in breast tumors (blood-based markers of stress-related tumor progression). The tissue specimens are collected for whole-genome gene expression analysis and the analysis of catecholamines and cAMP, and selected protein markers.

The collection of tissue specimens and blood will follow procedures that have been established for our protocol titled "Resource for the collection and evaluation of human tissues and cells from donors with an epidemiology profile", University of Maryland at Baltimore IRB #0298229. The same procedures are used in the ongoing lung

cancer and prostate cancer case-control studies. Our collaborating physicians will inform us of scheduled surgeries as soon as such a schedule becomes available and the day before surgery. Contract personnel will independently verify day and location of surgery. When the resection of a tumor is done, the OR will call the University of Maryland Department of Pathology. A designated person will transport the tissue to the pathology, where the tissue is procured as soon as possible. All biological samples will be stored at the pathology until further notice. The procedures are as follows.

Procedures for collection of blood

1. Observe universal precautions for prevention of transmission of blood-borne pathogens
2. Clean skin with alcohol wipe and wait to dry
3. Obtain blood: two 7 cc red top tubes and four 10 cc green top tubes. Apply pressure and band-aid

Procedures for processing of blood at the University of Maryland Department of Pathology

1. Blood (separation of serum and blood clot; buffy coat; plasma and red blood cells) to be processed within 8 hours at the University of Maryland Department of Pathology. Freeze about 20 million PBMCs in 5 million per vial aliquots

Procedures for tissue collection

1. Tissues are collected at time of mastectomy
2. Notify surgeon and the pathology department of tissue collection
3. A designated person will take tumor specimen as fast as possible to the pathology room
4. A pathology technician will immediately process the sample.
5. Within 30 min of receipt, place half of the specimen in a pre-labeled container. Flash-freeze and store the container at -70° C
6. Within 30 min of receipt, place about a quarter to a third of the specimen in a pre-labeled container and cover with OCT (for gene expression profiling). Flash-freeze the OCT-embedded tissue and store the container at -70° C.
7. Prepare a paraffin block of remaining tissue section
8. Prepare a H/E slide for diagnosis by pathologist

Assessment of disease stage and outcome

Information on disease stage, metastasis and outcome (relapse and survival) will be obtained from pathology and medical records, Veteran Administration databases, State of Maryland records, and from the National Death Index.

8. POWER CALCULATION

Required sample size for gene expression analysis

We will conduct a genome-wide gene expression analysis of breast tissues and compare expression profiles after stratifying patients into either low or high stress exposure (or tumor catecholamine levels) or using stratification into tertiles. The minimum number of tissues needed to generate a molecular profile that differentiates two groups (by stress exposure or tumor catecholamine levels) cannot be easily determined for the proposed experiments. A best estimate is 13 per group based on the following assumptions: A significance level of 0.001 and a power of 0.05, assuming the data is base 2log transformed and that the variance in expression for a gene of interest is 0.5, and that one wishes to be able to detect genes for which one of the following groups (all patients combined, AA patients, EA patients, patients with ER-positive tumors, patients with ER-negative tumors) exhibits a two-fold change in expression at the specified power (using nQuery software according to an analysis by Dr. Kevin Dobbin, NCI biostatistician).

Estimates for patient population

We assume that the recruited patient population in this study will consist of about 40% to 60% self-identified AA patients, with the other patients being mostly self-identified EAs and only few of the patients being self-identified Asian-Americans or Hispanic/Latinos, based on the breast cancer surgery schedules in previous years at UMMS and consistent with the general population in Baltimore City. The estimate is also consistent with the historical collection of tumor specimens from breast cancer patients by the NCI contractor covering the period between 1993-2004 (21,22). Thus, if we recruit 100 patients, an estimated 90 of them will be AA and EA patients and about half of those will be either an AA or an EA patient. Of those 100 patients, 30% to 40% will present with an estrogen receptor-negative disease, given us the statistical power us to analyze the influence of dichotomized stress exposure (perceived stress, discrimination-related stress, and perceived social isolation) on 1) gene expression

and catecholamine levels in breast tumors, 2) after stratification of patients by race/ethnicity (AA and EA), and 3) after stratification by tumor ER status. Without substratification by race/ethnicity, effects of stress in breast cancer subtypes can be analyzed (e.g., triple-negative tumors), which will require recruitment of the proposed maximum of 150 mastectomy patients for rare subtypes (here about 20-30 patients are expected to have the triple-negative disease in the study population). The exact same analysis can be done for dichotomized tumor catecholamine levels, identifying a gene signature associated with an above median tumor catecholamine level. A gene expression analysis based on a stress or catecholamine exposure comparing patients with the highest 25% versus lowest 25% exposure can be done for all breast tumors combined and for ER-positive tumors, and for ER-negative tumors and by race/ethnicity if we recruit the proposed maximum of 150 breast cancer patients. Lastly, a recently completed study of ovarian tumors indicated that a total sample size of n = 20 (10 per group after stratification) can yield a stress-related tumor gene signature (10).

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