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# INTRODUCTION

Breast cancer afflicts one in nine women by the age of 85 and is the second leading cause of cancer death among American women. The incidence has been increasing steadily in the United States to reach 182,000 new cases in 1993. Known risk factors account for only approximately a third of cases. It is highly likely that environmental factors (including exposures related to lifestyle, occupation and ambient pollution) are contributors, particularly in high risk areas such as the northeastern United States. Environmental contaminants such as the polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HA), cigarette smoke constituents and organochlorine residues are suspected mammary carcinogens of concern (1,2,3).

Traditionally, environmental cancer epidemiology has been hampered by difficulties in obtaining accurate data on individual exposures and on individual variation in response to carcinogens. The development of biomarkers has provided a tool that can circumvent these problems by providing individual measurements of the biologic dose of carcinogens, preclinical effects and susceptibility to cancer.

This project will determine: (1) whether specific environmental exposures are associated with PAH-, HA-, and smoking related-DNA adducts in mononuclear white blood cells and breast tissue); (2) whether these biomarkers are associated with breast cancer case-control status; and (3) whether increased carcinogen-DNA adduct levels are associated with the presence of mutations in the p53 tumor suppressor gene in breast tumors.

#### **1.** Environmental Exposures of Interest

Polycyclic aromatic hydrocarbons (PAHs) and aromatic amines are the two main classes of mutagenic chemical carcinogens that have consistently induced mammary tumors in experimental bioassays, and there is evidence that these compounds may play a role in human breast cancer development (3,4,1). <u>PAHs</u> are ubiquitous pollutants found in ambient air as well as the workplace environment, drinking water and food (5). Incomplete combustion of organic material, including fossil fuels, is the major source of PAHs, such as benzo(a)pyrene (BP), which is used as a representative indicator of total PAH concentrations (6).

Human exposure to <u>heterocyclic amines</u> comes principally through the diet. Creatine, amino acids, and sugars derived from muscle are important precursors for production of these mutagens (7). Muscle from meat, chicken, and fish produce similar mutagenic heterocyclic compounds, with temperature and time being the more important determinants of their formation during cooking (8).

Most studies of <u>active smoking</u> have found either a small positive association (about 20-30%) or no association with breast cancer (9,10,11,12,13,14,15). However, few studies have considered age of onset of smoking. A recent study has, in fact, shown that heavy smoking at an early age (before 16) is associated with a greater risk of breast cancer (odds ratio of 1.7) (9). There have been two reports of an increased risk of breast cancer from passive smoking. These results require confirmation (15). There is compelling evidence that constituents of cigarette smoke reach the breast and damage DNA through adduct formation (1,16,17).

# 2. Biomarkers Under Investigation: PAH-, HA-, Smoking related-DNA Adducts and p53 Mutational Spectra

It is apparent that epidemiologic studies have been severely limited by inadequate data on the amount, pattern and timing of environmental exposures, indicating a role for biomarkers in supplementing questionnaire and monitoring data. Extensive data indicate that most carcinogens, including PAHs, HAs and cigarette smoke constituents, are metabolically activated to electrophilic species capable of covalently binding to cellular macromolecules. In laboratory animals, the carcinogenic potency of a series of genotoxic carcinogens is generally correlated with their ability to form covalent adducts with DNA (18,19). Therefore, carcinogen-DNA binding is widely viewed as a necessary (though not sufficient) event in cancer induction. Adduct measurements can provide sensitive integrating dosimeters for potential mammary carcinogens. DNA adducts can be quantitated by the <sup>32</sup>P-postlabeling method which measures a broad spectrum of adducts on DNA (20). This technique can provide a measure of the amount of genotoxic carcinogen that is impinging on the target tissue, often referred to as the biologically effective dose, and can used as an exposure index in epidemiologic investigation. PAH-, HA-, and smoking related-DNA adducts are being analyzed in mononuclear white blood cells from cases, benign breast disease (BBD) controls, and healthy controls and in breast tissue from cases and BBD controls.

It has been suggested that mutational spectra in suitable reporter genes, such as <u>p53</u>, can reflect exposures to carcinogens that are strongly implicated in carcinogenesis (21,22,23). The spectrum of mutations found in these reporter genes can be conceptualized as the "finger print" left by mutagens that are likely to have contributed to the development of the cancer (24,22,23). <u>p53</u> is a tumor suppressor gene, the inactivation of which appears to play a critical role in carcinogenesis. In sporadic breast cancer, mutated <u>p53</u> has been found in approximately 50% of tumors (23,22). <u>p53</u> is thus a relevant reporter gene in which to analyze the effects of PAHs, HAs and cigarette smoke constituents on breast tissue.

Studies of the mutational spectra in breast cancer tumors have shown an increase in G $\rightarrow$ T transversions in CpG dinucleotides on the nontranscribed strand (22,23,25). G $\rightarrow$ T transversions appear to occur early in tumor development, and have been detected in all stages of disease with a prevalence of approximately 20% of all mutations(23,26,27). A similar mutational spectrum has been found in lung tumors for which environmental causes are well known (22,28). Combined with the fact that constituents of cigarette smoke (including PAHs) are known to cause G $\rightarrow$ T transversions, this knowledge has led to the suggestion that environmental factors may be responsible for the mutational spectra found in breast cancer (22,23). In addition, heterocyclic amines also known to induce G $\rightarrow$ T transversions (25). A finding of an association between PAH-, HA-, or cigarette smoke constituent-DNA adducts and p53 mutations in breast tissue would provide biologically meaningful evidence that these contaminants play a role in breast cancer development.

# 3. Preliminary Studies

A pilot study by Drs. Perera and Phillips, DNA adducts were detected in breast tissue samples by the <sup>32</sup>P-postlabeling method using the P1 nuclease extraction procedure (1). This method detects aromatic adducts including those formed by BP and other PAHs. Results were available from 31 specimens, including tumor and/or tumor adjacent tissue from 15 women with breast cancer and 5 control women undergoing reduction mammoplasty. Among cases, adduct levels ranged from 1.58 to 10.00 adducts/ $10^8$ nucleotides, with a mean of 4.69 adducts/ $10^8$  nucleotides in tumor tissue, 6.13 adducts/ $10^8$ nucleotides in tumor adjacent tissue and 5.3 adducts/10<sup>8</sup> nucleotides in tumor and tumoradjacent tissue combined. These values were in the lower end of the range seen in lung tissue of smokers and nonsmokers. Among controls adduct levels ranged from 0.43 to 4.41 adducts/10<sup>8</sup> nucleotides with a mean of 2.04 adducts/10<sup>8</sup> nucleotides. Smoking histories were available on the 15 cases. DNA samples from 5 of the 10 smokers (tumor and/or tumor adjacent tissue) displayed the characteristic pattern of smoking-related adducts (a diagonal zone of radioactivity) that has been reported in prior studies of lung cancer patients (29). None of the samples from the 5 nonsmokers showed this characteristic smoking-related pattern. This preliminary data indicated that PAHs reach breast tissue and cause genetic damage, and that the measurement of carcinogen-DNA adducts in breast tissue is a useful tool for the epidemiologic study of breast cancer development.

# 4. Study Design

The proposal calls for 100 breast cancer cases, 100 BBD (BBD) controls, and 100 healthy controls to be recruited in a case-control design. Cases and BBD controls are being recruited from the private practices of Drs. Estabrook and Schnabel at CPMC. Healthy controls are being recruited from the private Ob/Gyn practices of Drs. Hutcherson, Randolph and Clare at CPMC.

Controls are being frequency matched to cases on age and ethnic group (African American, Caucasian, Latina). Patients with conditions that are suspected of influencing blood biomarker levels independent to carcinogenesis are being excluded. Exclusion criteria include: prior history of cancer at any site, current pregnancy, breast feeding within the prior three months, and broken bones within the last six months. Within the BBD study group, patients with diagnoses of proliferative benign disease are being excluded. These diagnoses are associated with a increased future risk for breast cancer and these patients may share common risk factors with the case patients.

Blood samples, questionnaire data and pathology reports are being collected from all of the patients, and breast tissue samples are being collected from cases and BBD controls. Blood samples are being fractionated, processed and preserved for the assays to be conducted under this grant and to create a bank of specimens to support future research projects. Under this grant, mononuclear white blood cell (MWBC) samples will be analyzed for PAH-, HA-, and smoking related-DNA adducts using <sup>32</sup>P-postlabeling methods. The same carcinogen-DNA adducts will be assayed for in breast tissue specimens from cases and BBD controls. Additionally, breast tissue samples will be analyzed for mutations in the p53 tumor suppressor gene using the single strand conformation polymorphism assay and PCR sequencing.

Statistical analyses will be used to test our major hypotheses. Logistic regression analysis will be used to determine if carcinogen-DNA adduct levels measured in tissue and/or MWBC are associated with case-control status after controlling for confounding variables. Additionally, logistic regression will be used to test the hypothesis that among cases, mutations within the p53 tumor suppressor gene are associated with increased carcinogen-DNA adduct levels in tissue and/or MWBC. Finally, using questionnaire data on environmental, occupational and dietary exposures, associations between life-style factors and carcinogen-DNA adduct formation in MWBC and breast tissue will be investigated.

# BODY OF THE REPORT: PROGRESS DURING YEAR ONE 1. Patient Enrollment

Year one of the research project has been completed. This first year was devoted to establishing procedures for subject recruitment at the Columbia-Presbyterian Medical Center (CPMC). A successful system for identification and enrollment of cases and controls has been implemented in the private offices of Drs. Estabrook, Schnabel, Randolph, Hutcherson, and Claire. Cases and BBD controls are being enrolled under the direction of the breast surgeons, Drs. Estabrook and Schnabel. All patients undergoing breast surgery with these doctors are evaluated as potential subjects. Eligible patients are identified and enrolled after the physician has recommended surgery but before surgery is performed, generally one - two weeks later. Following enrollment, the patient is interviewed and a blood samples is drawn also prior to surgery to prevent confounding of biomarker data by exposures to anesthesia, chemotherapy, hormone therapy, biologic changes associated with the healing process, or post surgical changes in diet. The system for patient surveillance is critical to the successful identification and recruitment of patients during this one - two week period prior to surgery.

Healthy subjects are being enrolled under the direction of Drs. Randolph, Hutcherson and Claire through their private Ob/Gyn practices. Healthy controls are being frequency matched to cases on age and ethnicity. This design allows efficient statistical control of these potential confounding factors. Analysis of year one enrollment has shown that we have slightly under-enrolled healthy Caucasian subjects. In year two, we will focus on maintaining our enrollment of African American and Latina healthy controls and increasing our enrollment of healthy Caucasian subjects. We are evaluating the referral practices and patient populations of additional Ob/Gyn practitioners at CPMC. Doctors with suitable practices will be invited to participate in the study.

As a supplement to this study, we have received funds from the National Cancer

Institute to enroll patients through the Vanderbilt clinic system at CPMC. Using these funds, an active surveillance and recruitment system was set up in January of 1995 in the Vanderbilt Breast and Ob/Gyn clinics at CPMC. These clinics serve the Washington Heights, Northern Harlem, and Southern Bronx communities. These communities are largely African American and Latino (a large number of the Latinos are recent immigrants from the Dominican Republic). Recruitment is conducted with oversight by Drs. Krementz and Claire, directors of the two clinics. Every patient referred to surgery from the breast clinic is evaluated and those meeting the enrollment criteria are invited to join the study. Healthy controls enrolled from the Ob/Gyn clinics are being frequency matched on age and ethnic group to the clinic cases. Our program in these clinics has allowed us to enroll African American and Latina subjects of lower socio-economic status. This is a medically under served population that has not been included in past studies of breast cancer. Combined with the ethnic minority patients being enrolled through the above mentioned private practices, we expect by the end of the study, to achieve 50% minority recruitment.

As a result of these surveillance programs, patient enrollment has occurred at a faster pace than originally anticipated (see figure 1). This enhances the strength and validity of study in two ways. First of all, since we have a wide catchment system and are able to evaluate nearly every patient who enters it, we are better able to assure that we have a representative sample of cases. This oversampling will also assure that we will have complete data and samples (questionnaire and medical record data and tissue and blood samples) for 300 patients. The additional samples collected through these recruitment efforts will be stored for research projects to be conducted under separate funding.

| Category                                      | Patients |
|---|----------|
| Eligible Patients Contacted                   | 256      |
| Enrolled Patients                             | 184      |
| Cases   | 63       |
| BBD Controls                                  | 55       |
| Healthy Controls                              | 48       |
| Other*  | 8        |
| Subjects with as yet incomplete Path. reports | 10       |

Figure 1 Patient Enrollment

\* Includes lobular in situ, and phyloides tumors.

## 2. Laboratory Component

Blood samples have been collected from subjects and separated in total white blood cell, red blood cell, mononuclear white blood cell, and plasma components. In addition to preserving the blood samples for the assays funded under this proposal, our design called for storing of aliquots for future research. Sample aliquots have been processed and stored in anticipation of future analyses of; 1) organochlorines, 2) plasma vitamin C and E, retinoids and carotinoids, 3) hemoglobin adducts, 4) plasma erbB-2 extra-cellular domain, 5) plasma ras levels, 6) plasma p53 levels, 7) plasma EGFR levels, and 8) biomarkers of oxidative damage. This has created a sample bank that will allow future research to be conducted in an efficient and economical manner. As will be described below, this strategy is already paying dividends.

In reference to our Statement of Work, we are condensing the two sample shipments scheduled for year one into one large shipment at the end of the year. This will ensure that samples are analyzed in a single batch, and will reduce potential for laboratory variability and assay drift. Sending a shipment earlier would have been less methodologically sound, since samples would have come predominantly from the private patients.

It was originally proposed that fresh-frozen tissue would be assayed for carcinogen-DNA adducts. However, because fresh-frozen samples are not available from many needle localization biopsy procedures, or are too small for carcinogen-DNA adduct analysis, sections of the larger paraffin-embedded tissue blocks (which are available from all subjects) will also be utilized. Carcinogen-DNA adduct analysis will be conducted using the <sup>32</sup>P-postlabeling method as originally proposed (30). Adducts will be analyzed in paraffin-embedded tissue when fresh-frozen tissue is not available, and in both paraffinembedded and fresh-frozen tissue when fresh-frozen tissue is available.

## 3. Interviews and Pathology Reports

Each subject enrolled in the study has completed a structured interview covering demographic variables, accepted breast cancer risk factors, dietary sources of PAH and HA, smoking history, supplementary vitamin consumption, occupational history, residential history, family cancer history, environmental exposure history and alcohol consumption. The questionnaire has been translated into Spanish using vocabulary and idioms common to the Caribbean form of Spanish, which is the primary language spoken by the Latina patients seen at the CPMC clinics. All available pathology reports from our cases and BBD controls have been collected and reviewed. Information on tumor size, estrogen/progesterone receptor status, erbB-2 expression, DNA index, and proliferation index is being collected from the pathology reports. Currently, pathology reports have been reviewed for all but 10 patients enrolled. Pathology reports are not yet available for the 10 subjects, either because the Pathology Department has not completed their analysis, or because the patients need re-excisions to finalize the diagnosis. In addition, the database has been shaped and questionnaire and pathology report data are being extracted and computerized.

A preliminary review of our questionnaire data revealed that many of our Latina patients reported being extensively exposed to DDT while living in Dominica and other Central American and Caribbean nations. Subsequent interviews with Dominican officials revealed that there had been an extensive program of DDT spraying from 1946 until the mid-1960s, with intermittent spraying continuing into the 1990s. DDE, a metabolite of DDT, is suspected of playing a role in breast cancer development and this may be an exposure of concern for our Latina patients (2,31). In collaboration with Dr. Mary Wolff at Mount Sinai Medical Center, under separate National Cancer Institute funding, we are conducting a pilot study to analyze DDE levels in the stored blood samples from our Dominican patients. The results from this pilot will determine if these historical exposures have resulted in elevated blood DDE levels and whether this is a model population in which to efficiently investigate the role of DDE in breast cancer.

# CONCLUSION

The focus of this study is on discovering preventable environmental causes of breast cancer. Currently accepted risk factors explain only approximately 30% of breast cancer cases; most of these are not easily modulated through prevention programs. This study focuses on three classes of exposure (PAHs, HAs and cigarette smoke constituents) suspected in breast cancer etiology (3,4,1). To avoid some of the limitations of interview-based epidemiology, biomarkers of exposure (carcinogen-DNA adducts) and early effect (p53 mutations) have been incorporated into a hospital-based case-control study to facilitate evaluation of whether these exposures play a role in human breast cancer development.

A comprehensive system of patient identification and enrollment has been implemented which has enabled recruitment of subject from a representative spectrum of ethnic groups and socio-economic strata. Enrollment of patients and drawing of the blood samples prior to surgery has ensured that our biomarker data are not confounded by surgical and treatment exposures or by post-surgical changes in diet. Enrollment has been highly successful and patient accrual is occurring faster than originally anticipated. The increased enrollment will enhance our study, enabling detection of more subtle effects and assuring that a representative sample of cases has been enrolled.

As mentioned, preliminary inspection of interview data has revealed that large numbers of our Latina patients report heavy exposure to DDT, and presumably to its metabolite DDE. To our knowledge this is the first time that this potentially highly exposed cohort has come to the attention of public health researchers. It is believed that this population may be a useful public health model in which to evaluate the effects of DDE exposure on breast cancer development. The current evidence linking DDE exposure to breast cancer is controversial, the identification of a potentially highly exposed population may have substantial research significance. Under separate funding, a pilot study has been initiated to evaluate whether the reported heavy exposures to DDT has resulted in increased blood levels of DDE in the stored samples. The new research is possible because the U.S. Army funded parent study called for the preservation of plasma samples for future organochlorine analysis. This pilot research demonstrates the value of the blood and tissue sample bank and it is expected that additional spin-off research will be generated in the future.

A finding of an association between breast cancer and exposure to PAHs, HAs, or cigarette smoke could have significant public health implications. Intervention strategies, including education and regulation, are available, that could substantially reduce exposures to these ubiquitous pollutants. Since most currently recognized risk factors in breast cancer etiology are not readily amenable to modification through public health programs, prevention of breast cancer may be best realized through identification and reduction of environmental exposure contributing to the increased incidence in this disease.

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