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AUTHORITY
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GRANT NUMBER DAMD17-94-J-4251

TITLE: Biological Markers of Environmental Carcinogens in Breast Cancer

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Fort Detrick, Frederick, Maryland 21702-5012

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THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

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This ongoing case-control study is being conducted at the Columbia Presbyterian Medical Center (CPMC) and is investigating whether exposures to polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines (HAs), are associated with breast cancer. The study is designed to enroll 100 cases and 100 benign breast disease (BBD) controls from whom blood, biopsy tissue and questionnaire data are collected and 100 healthy controls from whom blood and questionnaire data are collected. The study is utilizing biomarkers (PAH-, HAs-, and smoking related-DNA adducts) as measures of exposure and p53 mutations as a biomarker of pre-clinical effects.

Patient recruitment has been completed with 119 cases, 107 BBD controls and 141 healthy controls enrolled. Laboratory assays for p53 in tissue sections and PAH-DNA adducts in white blood cells and tissue samples have been completed. Additionally, the study population has been analyzed for the GSTM1 genotype. Descriptive statistical analyses of the data are complete and we have begun analyzing the relationship between case-control status and biomarkers. Preliminary analyses show that after control for age, ethnicity, breast feeding, age at menarche, parity, smoking status, ETS exposure, and charred food consumption elevated tissue levels of PAH-DNA adducts are associated with breast cancer case-control status (OR = 2.7, p = 0.02).
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PI - Signature  1/19/99  Date
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INTRODUCTION

We have requested and been granted a one year no-cost extension for project DAAMD17-94-J-4251 "Biological markers of Environmental Carcinogens in Breast Cancer". The additional year will be used to complete the statistical analyses of the questionnaire and biomarker data. This report is an annual report for year four and includes a description of completed laboratory analyses and preliminary statistical analyses on PAH-DNA adduct levels in breast tissue and their association with case-control status. The final report will be submitted October 30th 1999 and will include a complete description of the results.

Breast cancer afflicts one in nine women by the age of 85 and is the second leading cause of cancer death among American women. In the U.S., the disease currently afflicts one out of nine women by the age of 85, with an estimated 178,700 new cases and 43,500 deaths in 1998 (1). It is 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 (PAH), heterocyclic amines (HA), cigarette smoke constituents and organochlorine residues are suspected mammary carcinogens of concern (2-4).

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, pre-clinical effects and susceptibility to cancer.

The goals of this project are to 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; (3) whether increased carcinogen-DNA adduct levels are associated with the presence of mutations in the p53 tumor suppressor gene in breast tumors; and (4) to store samples for the piloting of other biomarkers (e.g. ras p21, cyclin D1, erbB-2, DDE, and PCB) related to potential environmental and susceptibility factors in breast cancer.

1. Environmental Exposures of Interest

Polycyclic aromatic hydrocarbons (PAH) 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 (2,4-6). PAH are ubiquitous pollutants found in ambient air as well as the workplace environment, drinking water and food (7). Incomplete combustion of organic material, including fossil fuels, is the major source of PAH, such as benzo(a)pyrene (BP), which is used as a representative indicator of total PAH concentrations (8).

Human exposure to heterocyclic amines comes principally through the diet. Creatine, amino acids, and sugars derived from muscle are important precursors for production of these mutagens (9). 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 (10).

Most studies of active smoking have found either a small positive association (about 20-30%) or no association with breast cancer (11-17). 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) (11). There have been three reports of an increased risk of breast cancer from passive smoking (18). These results require confirmation (17,18). There is compelling evidence
that constituents of cigarette smoke reach the breast and damage DNA through adduct formation (see Preliminary Studies) (2;6;19;20). Recent work has seen a smoking associated risk in women who have a genetic polymorphism (NAT2 slow) which reduces their ability to detoxify aromatic amines (21). The effect was highest in women who began smoking prior to the age of 16.


Biomarkers can be used to supplement questionnaire and monitoring data. Extensive data indicate that most carcinogens, including PAH, HA 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, including PAH, is generally correlated with their ability to form covalent adducts with DNA (22;23). 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 \(^{32}\)P-postlabeling method which measures a broad spectrum of adducts on DNA (24). PAH-DNA adducts can also be analyzed by immunologic methods either by an Enzyme Linked Immunosorbent Assay (ELISA) or by immunohistochemistry (25-27). Here at Columbia, both methods utilize sensitive antibodies, developed in Dr. Regina Santella's laboratory at the Columbia School of Public Health, that recognize PAH-DNA adducts. These techniques 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 investigations. In the present study 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 \(p53\), can reflect exposures to carcinogens that are strongly implicated in carcinogenesis (28-30). The spectrum of mutations found in these reporter genes can be conceptualized as the "fingerprint" left by mutagens that are likely to have contributed to the development of the cancer (29-31). \(p53\) is a tumor suppressor gene, the inactivation of which appears to play a critical role in carcinogenesis. In sporadic breast cancer, mutated \(p53\) has been found in approximately 50% of tumors (29;30). \(p53\) is thus a relevant reporter gene in which to analyze the effects of PAH, HA 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 non-transcribed strand (29;30;32). \(G\rightarrow T\) 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 (30;33;34). A similar mutational spectrum has been found in lung tumors for which environmental causes are well known (29;35). Combined with the fact that constituents of cigarette smoke (including PAH) 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 (29;30). In addition, HA are also known to induce \(G\rightarrow T\) transversions (32). 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.

Other promising biomarkers include the oncogenes, \(ras\) \(p21\) and \(cyclin\ D1\), which are often overexpressed in breast tumors, and blood levels of the DDT metabolite (DDE) which has been associated with breast cancer development in some studies.
3. Preliminary Studies

In a pilot study by Drs. Perera and Phillips, DNA adducts were detected in breast tissue samples by the \( ^{32} \)P-postlabeling method using the P1 nuclease extraction procedure (2). This method detects aromatic adducts including those formed by BP and other PAH. Results were available from 31 specimens, including tumor and/or tumor adjacent tissue from 15 women with breast cancer and 5 healthy 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/\( 10^8 \) nucleotides in tumor and tumor-adjacent 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/\( 10^8 \) nucleotides with a mean of 2.04 adducts/\( 10^8 \) 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 (36). None of the samples from the 5 nonsmokers showed this characteristic smoking-related pattern. This preliminary data indicated that PAH 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. These findings have subsequently been confirmed by Li and colleagues (6).

4. Study Design

The current case-control study is designed to include 100 breast cancer cases, 100 BBD controls, and 100 healthy controls. Cases and BBD controls are being recruited from the private practices of Drs. Estabrook and Schnabel at Columbia-Presbyterian Medical Center (CPMC). Healthy controls are currently being recruited from the private GYN practices of Drs. Kelley and Levine 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 of carcinogenesis are being excluded. Exclusion criteria include: prior history of cancer at any site, current pregnancy, breast feeding within the prior three months, and bone fractures within the last six months. Within the BBD study group, patients with diagnoses of benign disease with atypia are being excluded. These diagnoses are associated with an increased future risk for breast cancer and these patients may share common risk factors with the cases.

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 are being analyzed for PAH-, HA-, and smoking related-DNA adducts and breast tissue samples are being analyzed for PAH-DNA adducts. Additionally, breast tissue samples are being analyzed for mutations in the p53 tumor suppressor gene using immunohistochemistry.

Statistical analyses are being used to test our major hypotheses. Logistic regression analysis is being 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 is being 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 are being investigated.
BODY OF THE REPORT: PROGRESS DURING YEAR FOUR

1. Patient Recruitment, Sample and Data Collection

   a. Patient Enrollment

   Patient enrollment was completed in June of Year four. Active patient surveillance programs were conducted in the offices of collaborating breast surgeons, Drs. Estabrook and Schnabel. Cases and BBD controls were enrolled by two interviewers under the direction of these two surgeons and their staff. All patients undergoing breast surgery with these doctors were evaluated as potential subjects. Eligible patients were identified and enrolled after the physician recommended surgery, but before surgery was performed. The study objectives and the patient's role in the study were explained to each of the prospective subjects and interested patients signed a consent form that met DOD and CPMC institutional requirements. Following enrollment, the patient was interviewed and a blood sample was drawn. Blood samples were drawn 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.

   Healthy control subjects were enrolled under the direction of Drs. Kelley and Levine and their staff. Dr. Kelley and Levine’s GYN practices are in the same building as the CPMC Breast Service and these doctors refer their patients to Drs. Estabrook and Schnabel for breast health care. Further, data on birth date and residential zip code were analyzed from a random sample of each physician's patients and were found to be similar across the physician's practices. Women were approached during routine GYN check ups with Drs. Levine and Kelley and were enrolled into the study. These women signed a consent form, donated blood samples and took part in the structured interview. Healthy control patients were frequency matched on age and ethnicity to cases.

   As a result of these surveillance programs, patient enrollment occurred at a faster pace than originally anticipated with a total of 417 patients enrolled (see Table 1). This over-sampling assured us that we would have complete data and samples (questionnaire and medical record data and tissue and blood samples) for a total of 300 subjects (100 cases, 100 BBD controls and 100 healthy controls).

<table>
<thead>
<tr>
<th>CATEGORY</th>
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<tr>
<td>Total Enrolled Patients</td>
<td>417</td>
</tr>
<tr>
<td>Cases</td>
<td>119</td>
</tr>
<tr>
<td>BBD Controls</td>
<td>108</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>141</td>
</tr>
<tr>
<td>Other*</td>
<td>46</td>
</tr>
<tr>
<td>Unknown**</td>
<td>3</td>
</tr>
</tbody>
</table>

* includes: benign breast disease with atypia, lobular carcinoma in situ, and rare cancers (e.g. Cystosarcoma phylloides).

** Complete pathology reports are still unavailable on for three subjects.
A total of 120 patients were asked to take part in the study but refused to enter it. Of these, 88 were prospective surgical enrollees and 32 were prospective healthy controls. Among surgical patients the response rate was 76% and among healthy subjects the response rate was 82% for an overall response rate of 78%. Non-responding surgical patients were on average older than the respondents, 60.8 years versus 53.1 years old, but had a similar ethnic distribution and the same prevalence of clinic versus private patients. Non-responding healthy controls were also older than responding healthy controls and were less likely to be Caucasian (29% of non-respondents were Caucasian, while 62% of respondents were Caucasian, p=0.001) and were less likely to be private patients (55% of non-respondents were private patients, while 79% of the respondents were private patients, p=0.005). This underscores the need to control for ethnicity in our statistical analyses (which in effect controls for private/clinic status since the two are highly correlated in our data).

b. Questionnaire and Pathology Data
Each of the patients took part in a structured interview that covers demographic variables, reproductive and health histories, diet, residential history, smoking, alcohol consumption, occupational history and environmental exposures. Interviews are conducted prior to surgery during the preoperative testing procedures. Data from the questionnaires were abstracted into a computer spreadsheet as soon as the interview is completed. The spread sheet is in MS Excel format for simple importation into the SPSS statistics package. Pathology reports and data on receptor, proliferative and clinical markers analyzed by the pathology department (estrogen/progesterone receptor status, erbB-2, DNA index, G0-G1, S, and G2-M cell cycle status) have also been collected and abstracted into the same spreadsheet as the questionnaire data. Additionally, information on stage and tumor size has been collected from the CPMC Tumor Registry. The data base has been cleaned and checked and we have begun statistical analysis.

c. Biological Specimen Collection and Storage
Blood samples were collected from subjects and separated into 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, 8) plasma cyclin D1, 9) biomarkers of oxidative damage, and 10) metabolic genotype (NAT2, GSTM1, CYP1A1). This has created a sample bank that will allow future research to be conducted in an efficient and economical manner. Under separate funding preliminary analyses of several of these markers have been conducted to support spin-off studies (see Additional Studies of Genetic Susceptibility and Oncoproteins).

Due to the small size of many of the lesions, frozen tissue is not available from all of our patients. Samples of DNA (50 ug) from the larger frozen samples are being collected. Paraffin embedded biopsy specimens from cases and BBD controls have been retrieved from the CPMC Pathology Department. Tissue sections from these specimen blocks have been cut with a microtome and stored on glass slides (10-20 slides for immunohistochemical analysis) or in plastic vials (for future DNA extraction). One slide from each patient was hematoxylin and eosin (H&E) stained to provide a histologic reference.

This archive of paired blood and tissue samples and associated questionnaire data and pathology reports is an invaluable resource that supports our current research and will form the basis of future projects that can be conducted in a timely and efficient manner.
2. Laboratory Component

The analyses of biological samples for PAH- and smoking related-DNA adducts is complete, as is the immunohistochemical analysis for p53 (see table 2). Descriptive analyses for these and other markers are presented below. The final analysis of the data set has begun, and we are currently investigating the relationship between adduct levels and possible confounding factors.

Table 2. Assays Completed for Major Hypotheses

<table>
<thead>
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<th>ASSAY</th>
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<tr>
<td>PAH-DNA in MNWBC</td>
<td>300</td>
</tr>
<tr>
<td>HA-DNA in MNWBC</td>
<td>150</td>
</tr>
<tr>
<td>Smoking Related Adducts in MNWBC</td>
<td>300</td>
</tr>
<tr>
<td>PAH-DNA in Fresh Tissue ((^{32})P)</td>
<td>18</td>
</tr>
<tr>
<td>PAH-DNA in Paraffin Tissue (IH)</td>
<td>298</td>
</tr>
<tr>
<td>P53 in Tissue (IH)</td>
<td>310</td>
</tr>
</tbody>
</table>

Abbreviations: MNWBC, mono-nuclear white blood cells; \(^{32}\)P, \(^{32}\)P-postlabeling; IH, immunohistochemistry.

a. Postlabelling Analysis of MWBC

Mononuclear white blood cell DNA was analyzed for the presence of PAH-, smoking related-, and heterocyclic amine-DNA adducts by \(^{32}\)P postlabelling methods in Dr. Phillips' lab. For PAH-DNA adducts results from a total of 300 subjects were returned to us from Dr. Phillips' lab. The mean adduct level is 5.40 adducts per \(10^8\) nucleotides with a standard deviation of 2.91 adducts. The data show a log normal distribution, consistent with our prior studies of PAH-DNA adducts in white blood cells. Each of the postlabelling chromatographs were analyzed for a diagonal radioactive zone (DRZ) which indicates the presence of smoking-related DNA adducts. Chromatographs from 35 of the 300 samples had smoking-related DNA adducts, which would be expected given the low prevalence of current smokers in the study population. None of the first 150 samples showed detectable levels of heterocyclic amine-DNA adducts and this assay was halted after year two.
b. PAH-DNA Adduct Analysis by Immunohistochemistry in Breast Tissue

We have used an immunohistochemistry assay to analyze the paraffin-embedded tissue samples for PAH-DNA adducts. The assay utilizes a sensitive polyclonal antiserum, developed in Dr. Regina Santella's laboratory at the Columbia School of Public Health, that is highly sensitive and specific for PAH-DNA adducts. Stained slides are analyzed on a Becton Dickson Cell Analysis System (CAS 200) which measures the Optical Density (OD) of the staining on the slides. The OD results provide a quantitative measure of the amount of antibody staining and thus of PAH-DNA adduct levels.

All of the available paraffin embedded tissue samples from cases and BBD controls have been assayed using this method. A total of 209 tumor and BBD tissue sections have been assayed with a mean OD of 0.42 and standard deviation of 0.253. Additionally, 89 tissue sections of normal tissue adjacent to the tumor have been analyzed. These sections had a mean OD of .430 and a standard deviation of .238. Among cases PAH-DNA adduct levels in tumor tissue and normal adjacent were correlated, with the OD scores for the 85 paired tissue samples, having an \( r=0.61, \ p<0.001 \).

We have begun case-control analyses of the tissue PAH-DNA adduct results, and the preliminary results are outlined below. Case tumor samples had a mean OD of 0.47 and BBD control samples had a mean OD of 0.38, \( p=0.1 \). Adduct levels were dichotomized into high versus low using a cutoff of one standard deviation above the mean level seen in the BBD controls. In univariate analyses the presence of high levels of PAH-DNA in breast tissue was positively associated with breast cancer status, OR = 2.4, \( p=0.02 \). After controlling for potential confounding by ethnicity, age, smoking status, ETS exposure, age at menarche, parity, consumption of charbroiled food and breast feeding, the presence of high levels of PAH-DNA adducts in breast tissue was positively associated with breast cancer status, OR = 2.76, \( p=0.02 \).

c. p53 Analysis

Immunohistochemical techniques have been used to initially screen for p53 mutations. While wild type p53 has a very short half-life, many mutant p53 proteins have an increased stability leading to an accumulation of protein that is detectable using immunohistochemical techniques (30;37). Immunohistochemical detection has been found to correlate well with SSCP/PCR techniques for mutant detection (38-41).

In the literature samples are scored positive or negative for p53 accumulation based on the intensity of staining, the percentage of cells that stain positive for p53, or a combination of these two measures (33;38;42;43). Both of these indexes can be measured on the CAS 200 system by using the Quantitative Nuclear Antigen Program (QNAP). QNAP incorporates a thresholding system which determines the percentage of nuclei that have been stained darker than a certain optical density level. This threshold is set by determining the optical density of nuclei that have been assayed using an immunohistochemical protocol that omits the primary p53 antibody. This provides an objective measure of the percentage of cell nuclei that have been stained darker than the background level of the assay.

Immunohistochemical analysis has been completed on all the tissue sections received from the Pathology Department. From the benign breast disease subjects, 105 samples analyzed and from the cases, 105 normal adjacent and 100 tumor samples were analyzed. Of the cases, 28% of the tumor samples showed greater than 15% of the nuclei staining positive for p53. In both the normal adjacent and benign samples 8% of the tissue samples showed greater than 15% of the nuclei staining positive for p53. Further statistical analyses are being performed to determine whether PAH-DNA adduct levels in tissue or blood are associated
with p53 status. Additionally we will analyze the questionnaire data to determine whether environmental exposures such as cigarette smoke are associated with p53 status.

d. Additional Studies of Genetic Susceptibility and Oncoproteins

Over the course of the study we have used funds available from other sources to analyze stored tissue and blood samples for other markers such as: genetic susceptibility, exposure to organochlorine compounds and various oncoproteins (see Table 3.). These markers were chosen either because they would complement the markers used in our main study or for use as pilot data to support additional grant proposals. In year four we continued our work on GSTM1 and cyclin D1.

Table 3. Assays Performed for Complementary and Pilot Studies

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>COMPLETED IN YEAR 2</th>
<th>COMPLETED IN YEAR 3</th>
<th>COMPLETED IN YEAR 4</th>
<th>TOTAL</th>
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<tr>
<td>NAT2</td>
<td>120</td>
<td>0</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>GSTM1</td>
<td>134</td>
<td>0</td>
<td>231</td>
<td>365</td>
</tr>
<tr>
<td>Cyp1A1 MSP1</td>
<td>124</td>
<td>0</td>
<td>0</td>
<td>124</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>41</td>
<td>51</td>
<td>58 (blood)</td>
<td>150</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>42</td>
<td>31</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>Ras p21</td>
<td>94 (blood)</td>
<td>0</td>
<td>0</td>
<td>94</td>
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<tr>
<td>Organochlorines¹</td>
<td>0</td>
<td>1,200</td>
<td>0</td>
<td>1,200</td>
</tr>
</tbody>
</table>

¹ Includes analyses for DDE, DDT, 21 different PCB congeners, hexachloro-benzene and Mirex on 48 subjects.

i. Complementary Markers: Polymorphisms in Metabolic Genes

Over the course of the study we have analyzed white blood cell DNA from our breast cancer cases and controls for polymorphisms in NAT2, GSTM1 and CYP1A1, genes that mediate the metabolism/detoxification of the environmental carcinogens under study (44-50). In year four we completed the analysis of polymorphisms in the GSTM1 gene in all of our subjects who donated blood. The null GSTM1 genotype has been associated with increased PAH-DNA adduct levels, appears to play a role in detoxifying cigarette smoke constituents and is thought to be associated with an increased risk for breast cancer (51-55). Of the 365 samples assayed, 333 could be successfully amplified by PCR. The at risk null genotype was found in 51% of our subjects. This genotype data set will be combined with our case-control and adduct data bases and will be used to analyze possible gene-environment interactions in breast cancer development.

ii. Pilot Studies: Development of a Western Blot Assay to Detect Cyclin D1 In Blood Samples

The Cyclin D1 gene is thought to be a proto-oncogene, which, when aberrantly expressed, leads to
loss of normal growth control (56;57). *Cyclin D1* overexpression is commonly seen in squamous cell carcinomas of the head, neck, lung and esophagus, as well as in breast carcinomas (56-59). Using immunohistochemical techniques, *cyclin D1* protein overexpression can be detected in 50-60% of paraffin embedded breast tumor specimens, and overexpression is associated with estrogen receptor positive tumors and a better prognosis (57;59;60;60).

In years two and three, 67 tissue sections from 25 BBD controls and 23 cases were assayed for cyclin D1 expression. Blood plasma samples were available from 24 of the BBD controls and 19 of the cases; and in year four these were analyzed for the presence of cyclin D1 using a newly developed Western blot assay. Additionally, blood plasma samples were analyzed from 15 healthy controls who were individually matched on age and ethnicity to the first 15 cases. Cyclin D1 was detected in 21 of the blood samples, with the following distribution among cases and controls: 3 of 15 healthy controls (20%), 6 of 24 of BBD controls (25%), and 12 of 19 cases (63%), p=0.011 by Chi-square. The odds ratio for *cyclin D1* and breast cancer (cases versus BBD controls) was 5.14, 95% C.I. 1.38-19.12 (p=0.012) and for cases versus healthy controls it was 6.86, 95% CI 1.43-33.01 (p=0.012). There was no correlation between the presence of cyclin D1 in blood plasma and the level of cyclin D1 expression in tumor tissue, however this analysis was limited by the small number of subjects.

Since the Western blots were scored for the presence of absence of cyclin D1 bands with the unaided eye, it is not possible to determine objectively whether the bands seen in the controls were lighter or smaller than the bands seen in cases. We are currently developing an ELISA assay for cyclin D1 that will give us a quantitative measure of the protein concentration in the blood samples. We expect that such a quantitative measure will allow us to set a cutoff concentration that will better discriminate between cases and controls. This strategy has successfully been used to assay for blood levels of the erbB-2 extracellular domain (see for example (61)).

Several grant proposals have been submitted to further develop this assay and validate cyclin D1 in blood as a marker of breast cancer status. Such a bloodborne tumor derived marker would be of great use in the early detection of breast cancer. Further, the relationship between cyclin D1 overexpression in tumor tissue and estrogen receptor status and prognosis indicate that a blood marker for cyclin D1 may be of great use in providing pre-surgical information about the tumor. Lastly, serial blood sampling for cyclin D1 after surgery may also be of great use in patient follow-up and management.

**CONCLUSION**

The fourth year of the study has been highly productive, with all of the laboratory analyses of the specimens completed and with the important addition of the complete analysis of GSTM1 genotype. This work, along with questionnaire data, provides us with information on exposure to PAH, the bioeffective dose of PAH, a marker of genetic susceptibility related to PAH, a marker of pre-clinical effect important in breast cancer development, and disease status. Preliminary analyses indicate that the presence of high levels of PAH-DNA adducts are associated with case/BBD control status and that confounding factors such as known breast cancer risk factors and exposure to cigarette smoke cannot explain these results. Further analyses in year five (unfunded) will assess whether PAH exposure and PAH-DNA adduct formation is associated with p53 mutations. Further, we will determine whether the deletion of GSTM1 is associated with increased PAH-DNA adduct levels in the breast and whether deletion is associated with p53 status. This work should provide valuable information on the potential environmental etiology of breast cancer and may suggest new preventive strategies.
References


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MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@dot.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for Information Management
Reports to be changed to "Approved for public release; distribution unlimited"

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