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The relationship between genetic damage from polycyclic aromatic hydrocarbons in breast tissue and breast cancer

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A number of polycyclic aromatic hydrocarbons (PAH) are widespread environmental contaminants that cause mammary cancer experimentally. We investigated whether exposure and susceptibility to PAH, as measured by PAH–DNA adducts in breast tissue, are associated with human breast cancer. We carried out a hospital-based case-control study using immunohistochemical methods to analyze PAH–DNA adducts in tumor and nontumor breast tissue from cases and benign breast tissue from controls. The subjects were white, African-American and Latina women without prior cancer or treatment, including 119 women with breast cancer and 108 with benign breast disease without atypia. PAH–DNA adducts measured in breast tumor tissue of 100 cases and in normal tissue from 105 controls were significantly associated with breast cancer (OR = 4.43, 96% CI 1.09–18.01) after controlling for known breast cancer risk factors and current active and passive smoking, and dietary PAH. There was substantial interindividual (17-fold) variability in adducts overall, with 27% of cases and 13% of controls having elevated adducts. The odds ratio for elevated adducts in tumor tissue compared with control tissue was 2.56 (1.05–6.24), after controlling for potential confounders. Adduct levels in tumor tissue did not vary by stage or tumor size. Among 86 cases with paired tumor and nontumor tissue, adducts levels in these two tissues were highly correlated (r = 0.56, $P<0.001$). However, the corresponding associations between case-control status and adducts measured in nontumor tissue from 90 cases and in normal tissue from 105 controls were positive but not statistically significant. Overall, neither active nor passive smoking, or dietary PAH were significantly associated with PAH–DNA adducts or breast cancer case-control status. These results suggest that genetic damage reflecting individual exposure and susceptibility to PAH may play a role in breast cancer; but more research is needed to determine whether the findings are relevant to causation or progression of breast cancer.

Abbreviations: BBD, benign breast disease; BPDE, benzo[*a*]pyrene diol epoxide; CPMC, Columbia Presbyterian Medical Center; DCIS, ductal carcinoma *in situ*; GSD, geometric standard deviation; OD, optical density; PAH, polycyclic aromatic hydrocarbons.

Introduction

In the United States, one out of nine women will receive a diagnosis of breast cancer by the age of 85, with an estimated 178 700 new cases and 43 500 deaths in 1998 (1). In recent decades, the incidence of breast cancer has increased by about 1% per year in the US (2,3). Because known risk factors account only for an estimated 40–50% of breast cancer cases in the United States (4) there is growing interest in the hypothesis that environmental contaminants may be playing a causative role (5–10). It is anticipated that the identification of environmental risk factors will offer new opportunities for prevention.

Polycylic aromatic hydrocarbons (PAH) are an important class of chemical carcinogens that are widespread in the ambient environment due to fossil fuel combustion for energy production, transportation and industry (11). PAH are also found in tobacco smoke and foods such as charred and broiled meat (11,12). A number of PAH are potent mammary carcinogens in experimental bioassays (6,13). *In vitro* studies show that human breast epithelial tissue has the ability to metabolize PAH to their ultimate mutagenic/carcinogenic moieties (14–16) capable of forming PAH–DNA adducts in human breast tissue. Carcinogen–DNA adducts are considered a necessary, but not sufficient, event in the development of malignancy (17). Perera and colleagues reported the occurrence of PAH– and other aromatic–DNA adducts in breast tumor and nontumor tissue in a study of breast cancer cases and mammoplasty controls using ^{32}P-postlabeling with the P1 nuclease extraction procedure as the method of detection (5). A subsequent study, also using the ^{32}P-postlabeling method, assayed nontumor tissue adjacent to tumor tissue from 89 breast cancer patients, as well as normal tissue from 29 reduction mammoplasty patients, and found cases to have significantly higher adduct levels than controls (18). A limitation of both studies is that women seeking reduction mammoplasties do not constitute a representative sample of the source population from which the cases were derived, and are not epidemiologically sound controls. Reduction mammoplasty patients are likely to be more affluent, to have larger breasts, to be younger and to have increased health care seeking tendencies than other women in the source population. Moreover, neither study controlled for possible confounding by known risk factors for breast cancer. The current investigation was designed to address both of these issues.

In 1994, we initiated a molecular epidemiologic case-control study of breast cancer at the Columbia Presbyterian Medical Center (CPMC) to test the hypothesis that exposures to PAH play a role in breast cancer development. Particular attention was paid to control selection and to gathering interview data on known and suspected breast risk factors. PAH–DNA adducts were measured in breast tissue from cases and controls using an established immunoperoxidase assay with the monoclonal

antibody 5D11 (19,20). This and similar immunohistochemical assays have been used to analyze a wide variety of human tissues for DNA damage (20–29). The advantages of this assay are that it can be used with small tissue samples, adduct levels in adjacent morphologic structures in the same tissue can be evaluated, it has good sensitivity and specificity for measuring DNA adducts, and it can be applied to fixed paraffin embedded samples (30). The immunohistochemical assay using the monoclonal antibody 5D11 has been applied in several prior published studies of aorta tissue samples, ovarian granulosa-lutein cells, sperm and human embryos (19,23,24,31). This work has shown consistent relationships between staining intensity and smoking status. For example, in recent studies of ovarian granulosa-lutein cells using this assay with the antibody used in the present study, both active and passive smokers had higher staining for PAH–DNA adducts than nonsmokers; and staining intensity correlated with follicular fluid cotinine levels and with cigarettes smoked per day (31).

Materials and methods

Study population

From 1994 to 1998, women with breast cancer and women with benign breast disease without atypia were enrolled into a hospital based case-control study at CPMC. Patients who were referred for breast surgery without prior cancer or prior treatment were recruited prior to treatment through private practices and the breast clinic. Undergoing a screening mammogram or participation in a breast cancer screening program were not eligibility criteria. After informed consent had been obtained and during their preoperative tests, patients took part in a structured interview covering established reproductive breast cancer risk factors, active and passive smoking, dietary practices, other environmental and occupational exposures, and vitamin consumption. Patients whose confirmed diagnosis was of ductal carcinoma *in situ* (DCIS) or invasive ductal or invasive lobular cancer were defined as cases. Women with rare histologic types such as cystosarcoma phyloides or mucinous tumor were excluded due to potential etiologic heterogeneity and because their small numbers precluded subgroup analyses. Patients diagnosed with benign breast disease or benign breast disease with hyperplasia were classified as controls. Patients with diagnoses that did not fit these categories (e.g. benign breast disease with atypia or lobular carcinoma *in situ*) were excluded from analysis because of their elevated risk of future breast cancer. Thus the control group represents women at average or only slightly elevated risk for future breast cancer and constitutes a representative sample of the joint source population of women seen at the CPMC Breast Service.

Breast cancer patients seen at CPMC for follow-up surgery (e.g. mastectomy or re-excisions) after an initial surgical biopsy at another hospital were excluded from the study. Additional exclusion criteria included a prior history of cancer at any site except basal skin cancer, age of <35 or >75 years, current pregnancy, recent bone fractures, or recent breastfeeding. The last two exclusion criteria were included because these factors might interfere with other biomarkers measured in this study.

The study protocol was approved by the CPMC Institutional Review Board and by the Columbia Comprehensive Cancer Center's Review Board. All subjects signed an informed consent form.

Laboratory methods

Before paraffin-embedded tissue blocks were released to the laboratory for slicing, the blocks and pathology reports blocks were re-reviewed by a single pathologist. This provided a consistent pathology review and ensured that the blocks contained representative tissue samples. Using a microtome, 5 μm slices were cut from the blocks and prepared on glass slides for immunohistochemical analysis. Benign tissue was available from the controls, while samples of both tumor and histologically normal tissue were available from the cases. Tumor tissue samples from 100 cases, normal tissue samples from 90 cases, and tissue samples from 105 controls were of adequate size and quality for PAH–DNA analysis. Blood samples (30 ml) were drawn from all women at enrollment for analyses of additional markers not reported here. All samples were coded.

The immunohistochemical assay for PAH–DNA adducts was carried out as described (19,31). Tissue slides were washed with PBS twice, treated with RNase (100 μg/ml, Sigma Chemical Co., St. Louis, MO) at 37°C for 1 h, washed with PBS, treated with proteinase K (10 μg/ml, Sigma Chemical Co.) at room temperature for 7 min and then washed. To denature the DNA, slides were incubated with 4 N HCl for 7 min at room temperature and then with 50 mM Tris base for 5 min at room temperature. After washing the slides with PBS, nonspecific binding was blocked with 1.5% normal horse serum and slides were incubated overnight at 4°C with anti benzo[*a*]pyrene diol epoxide (BPDE)-DNA monoclonal antibody 5D11 (1:50 dilution in normal horse serum) kindly provided by Dr Regina Santella. On the following day, the slides were incubated with biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 30 min at 37°C. Endogenous peroxidase was blocked by treating the slides with 0.3% H_2O_2 in methanol for 15 min at room temperature. Slides were rinsed in 1% Triton X-100 for 30 s before incubation with ABC reagent (Vector Laboratories) for 30 min at 37°C. After washing with PBS, diaminobenzidine (Vector Laboratories) solution was added for 3 min at room temperature. Methyl green (Sigma Chemical Co.) was used as a counterstain. Slides were dehydrated, cleaned in serial dilutions of ethanol and xylene and mounted with Permount (Fisher Scientific, Pittsburg, PA).

Staining was quantified by absorbance image analysis using a Cell Analysis System 200 microscope (Becton-Dickinson, San Jose, CA) running the Cell Measurement Program. Absorbance of light at a wavelength of 500 nm was measured because methyl green does not absorb light at this wavelength while diaminobenzidine does. A total of 50 epithelial cells (five fields with 10 cells per field scored) were measured on each tissue slide and results are reported in Optical Density (OD) units as previously described (19). The scored cells were selected to be representative, in terms of intensity, of the cells in the field and a single technician scored all of the samples. The reliability of the scoring procedures was assessed by having a second technician re-score a randomly selected subset of the tissues samples (tumor $n=15$, nontumor $n=15$, benign $n=15$). Serial tissue slices from laboratory control breast tissue specimens previously shown to have low and high staining for adducts were used as negative and positive control samples respectively, and were run with every batch. As an additional negative control, in each batch a laboratory control sample was run without the primary antibody. The coefficient of variability was assessed for the series of positive and negative control samples and was 20% for the positive control series and 29% for the negative control series. This represents the total variability due to intra-tumor differences, lab variability and scoring variability and compares favorably with the coefficient of variability of other methods for assessing adduct levels (32).

The immunohistochemical assay for PAH–DNA was initially tested using a polyclonal antibody to stain cells treated with high levels (5–40 μM) of BPDE, and this work showed increasing staining with increasing dose (33). More recent studies using immunohistochemistry with the 5D11 antibody and cells treated with BPDE (3.7–74 μM) have also shown a strong correlation ($r=0.99$, $P=0.011$) between treatment dose and staining intensity (24,31). Studies of assay specificity for both the monoclonal and polyclonal antibodies found that preabsorption of the antibody with BPDE-DNA, or pretreating the cells with DNase, or running the assay without the primary antibody all reduced staining to background levels (19,33). Although the 5D11 antibody was produced in response to the adduct of the carcinogenic intermediate of benzo[*a*]pyrene (BPDE-DNA), it crossreacts with varying affinities with other structurally related PAH; hence the terminology 'PAH–DNA' is commonly used (30,33,34). Previous results from this and similar studies using immunohistochemical assays to measure PAH–DNA adducts have therefore been reported in OD units (22,27,33,35). Here also, OD levels provide a scale reflecting the relative intensity with which the tissue sections were stained, as a means to compare the subjects in terms of PAH–DNA adduct levels in breast tissue.

Statistical analyses

Potential differences in demographic variables between respondents and nonrespondents and between cases and controls were analyzed by Student's *t*-tests for continuous data and χ^2 for categorical data. Because the distribution of PAH–DNA adducts was log normal, the OD data were natural log transformed prior to *t*-tests, ANOVA, and regression. OD data are reported both as arithmetic and geometric means to be consistent with other studies and to allow future investigators accurately to calculate power for adduct based studies in breast tissue. Reliability analyses for the scoring procedures were performed using interclass-correlation analyses of natural log transformed data assuming that the technicians were randomly selected from a population of possible technicians (36).

Logistic regression analyses were performed to assess the associations between known and suspected breast cancer risk factors (shown in Table I) and case-control status. The odds ratios were compared with those in the literature to assess whether the use of benign breast disease controls had introduced a bias into the study. Student's *t*-tests were used to determine whether the mean adduct level differed between cases and controls. Logistic regression was used to calculate the odds ratios for the association between each unit increase in OD level and case-control status. A comparison of data

Table I. Comparison of source, ethnicity, and age in surgical enrollees and nonrespondents

	Number of subjects (%)	
	Surgical enrollees	Nonrespondents[a]
Source		
Private	184 (81)	69 (82)
Clinic	43 (19)	15 (18)
Ethnicity		
Caucasian	149 (66)	43 (66)
Non-Caucasian	78 (34)	22 (34)
Mean age (years)	53.16	55.42

[a]Women who were invited to participate but declined.

distributions in tumor tissue from cases and benign tissue from controls suggested that cases were more likely to be in the upper tail of the OD distribution (Figure 1). Thus, subjects were dichotomized into high versus low adduct level groups using a cutoff of the mean plus one standard deviation in the benign tissue from controls. A similar approach to setting a cutoff was used in a prior lung cancer case-control study of adducts (37). Analyses were performed first on data from tumor tissue data versus benign tissue and then on data from nontumor tissue from cases versus benign tissue. Potential confounding variables (age, parity, age at first birth, age at menarche, ethnicity, breast feeding status, family history of breast cancer, and alcohol consumption) were included in the logistic regression models. Complete questionnaire data were not available from all of the subjects for whom PAH–DNA adduct data were available. Thus, the number of subjects included in analyses that involved questionnaire data is slightly lower than the number reported in raw analyses of the PAH–DNA adduct data.

In order to consider the role of individual susceptibility to environmental carcinogens, the final logistic regression models included both PAH exposure variables [current smoking status, current environmental tobacco smoke (ETS) exposure and charred meat consumption] and breast cancer risk factors. Because PAH–DNA adduct measurements represent the joint effects of exposure and interindividual variation in metabolism and DNA repair, when adjusted for current exposure, adducts are expected to reflect individual variation in metabolism and DNA repair (37, 38).

Since the biomarker-disease relationship may differ by menopausal status (39), analyses were also performed within pre- and post-menopausal strata. The benign breast disease control group had a higher prevalence of women with a family history of breast cancer than might be expected in a healthy control group. To assess the extent to which this might have biased the analyses of adduct levels, the data were reanalyzed in the subgroup of women without a family history and also in the women aged 40 years or older.

The study was not designed with a sufficient sample size to detect the modest odds ratios generally associated with questionnaire data on smoking and other exposure variables. However, because these variables were included in logistic regression models as potential confounders, odds ratios were calculated and are reported.

Possible determinants of PAH–DNA adduct levels in each tissue type were evaluated, including sources of PAH such as tobacco smoke and charred food. Student's *t*-tests were used to determine whether PAH–DNA adduct levels as a continuous variable were associated with dichotomous variables such as current smoking status and current passive smoke exposure. ANOVA and trend tests were used to determine whether PAH–DNA adduct levels were associated with categorical variables such as daily cigarette consumption (none, <median, >median) in the past year. Finally, the associations between PAH–DNA adduct levels and possible predictor variables were assessed using a multiple linear regression model that included ethnicity, menopausal status, current smoking status, current ETS exposure, consumption of charred meats, and estrogen receptor levels (percentage of cells staining positive for the receptor). Estrogen receptor levels were neither measured in nontumor tissue from cases nor in benign tissue from controls; and data were not available from women with DCIS and from some women with invasive cancer. Therefore, separate models were implemented for tumor tissue data with and without the estrogen receptor variable.

Data are presented in Tables I–IV, with P values reported when $P \leq 0.05$.

Results

A total of 401 patients who appeared eligible for the study were referred to surgery during the enrollment period. During the pre-surgical enrollment procedures, 39 patients (10% of potential subjects) were found to be ineligible (e.g. were found to have a prior history of cancer or a recent bone fracture) and were excluded from the study. A total of 88 subjects (24% of the eligible patients) refused to enter the study, mainly due to anxiety about the upcoming surgery. Another 47 subjects (13% of the eligible patients) were excluded because of a final diagnosis of atypia, lobular carcinoma *in situ*, or a rare breast

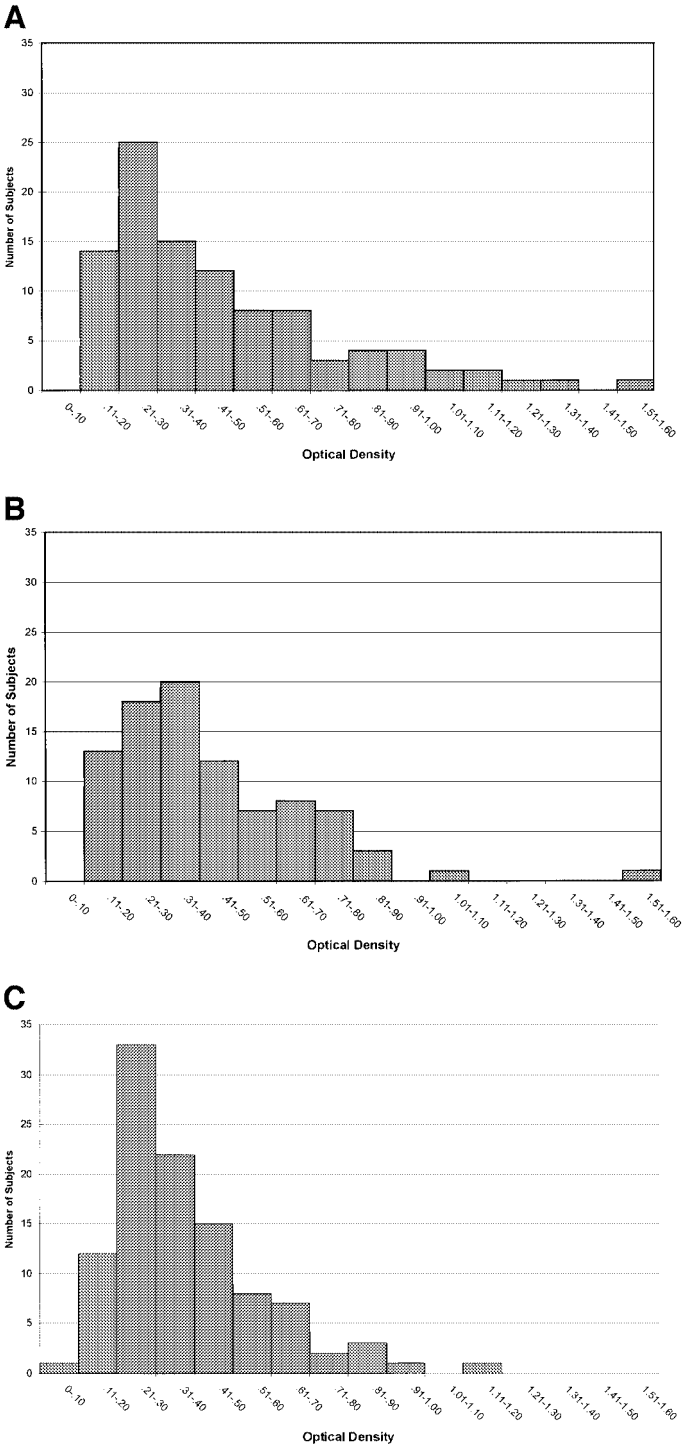

Fig. 1. (A) Distribution of staining intensity as measured by optical density scores in tumor tissue from cases. **(B)** Distribution of staining intensity as measured by optical density scores in nontumor tissue from cases. **(C)** Distribution of staining intensity as measured by optical density scores in benign tissue from controls.

Table II. Demographic and reproductive characteristics of cases and controls

	Controls (n = 108)[a]	Cases (n = 119)[a]	Odds ratio[b] (95% confidence limit)
Mean age (years)[c]	49.6	56.2	1.07 (1.04–1.11)
SD	8.9	10.5	
	n=108	n=119	
Ethnicity[c]			
Non-Caucasian	n=48 (44%)	n=30 (25%)	1
Caucasian	n=60 (56%)	n=89 (75%)	2.04 (1.02–4.06)
Regular current alcohol consumption[c]			
No	n=58 (55%)	n=49 (42%)	1
Yes	n=47 (45%)	n=69 (58%)	1.95 (1.05–3.62)
Family history of breast cancer			
No	n=85 (82%)	n=93 (80%)	1
Yes	n=19 (18%)	n=23 (20%)	0.87 (0.40–1.89)
Breast feeding history			
Never	n=60 (57%)	n=63 (53%)	1
Ever	n=45 (43%)	n=56 (47%)	1.01 (0.50–2.02)
Age at menarche			
<13 years	n=53 (50%)	n=60 (50%)	1
>13 years	n=53 (50%)	n=59 (50%)	1.05 (0.57–1.92)
Parity			
Parous	n=82 (77%)	n=103 (87%)	1
Non-parous	n=24 (23%)	n=16 (13%)	0.47 (0.19–1.14)
Mean age at first birth (years)	24.6	25.8	1.04 (0.94–1.12)
SD	4.8	4.5	
	n=80	n=103	

[a]Complete questionnaire data were not available from all of these subjects, so the n for some variables is smaller than the total number of enrolled subjects.
[b]Controlling for all other variables in the Table.
[c]$P < 0.05$.

cancer type. Of the remaining subjects, 119 and 108 women had confirmed diagnoses corresponding to the case and control definitions, respectively. The cases were predominantly of early stage cancer (17% DCIS, 46% stage I, 34% stage II, and only 3% stage III). Analysis of available data on age and whether the patient was drawn from the clinic or private practice showed that non-respondents did not differ from the surgical enrollees (Table I).

The demographic distributions for enrolled cases and controls differed somewhat and, as expected, reflected the established risk factors for breast cancer (40) (Table II). Consistent with the literature, increasing age, Caucasian ethnicity and regular current alcohol consumption were significantly and positively associated with case-control status (40,41) (Table II).

PAH–DNA adduct data in breast tissue were available from 104 cases (100 tumor samples, 90 nontumor samples) and from 105 controls. Potential confounding variables, such as age, age at menarche, parity, menopausal status, age at menopause, and age at first birth, were compared in subjects for whom PAH analysis was performed versus those subjects for whom PAH analysis could not be performed; no significant differences were found.

Figure 1 shows the distribution of adducts in tumor and nontumor tissue from cases and in benign tissue from controls. Figure 2 shows PAH–DNA adduct staining results from (A) a case with stage 1 breast cancer whose tumor tissue shows strong dark staining, and (B) a case with stage 2 breast cancer whose tumor shows weak staining. The scoring procedures proved to be very reliable with interclass correlation coefficients of 0.80 ($P = 0.0001$) for tumor tissue, 0.72 ($P = 0.0006$) for benign tissue and 0.93 ($P < 0.0001$) for nontumor tissue.

The unadjusted arithmetic mean adduct level was significantly higher for the tumor tissue samples (mean = 0.47, SD = 0.30) compared with the benign tissue samples (mean = 0.38, SD = 0.19, $P = 0.02$), and marginally higher in nontumor samples from cases (tissue mean = 0.43, SD = 0.24) versus those from benign controls (mean = 0.38, SD = 0.19, $P = 0.14$). Reflecting the right-skewed distribution of adducts, unadjusted geometric mean adduct levels were only slightly higher in tumor samples from cases compared with controls (geometric means = 0.39, GSD = 1.84 and geometric mean = 0.34, GSD = 1.60 respectively, $P = 0.1$) and in nontumor tissue from cases versus controls (nontumor tissue geometric mean = 0.37, GSD = 1.67 versus mean = 0.34, GSD = 1.60, $P = 0.2$). Stratification by menopausal status did not alter the case-control differences. Regression analysis showed that adduct levels in paired tumor and nontumor tissue from breast cancer patients were significantly correlated (slope = 0.65, r = 0.56, $P < 0.001$ for 86 subjects) (Figure 3).

When adducts measured in tumor tissue and tissue from benign controls were considered as a continuous variable, the odds ratio was 4.05 (95% CI 1.26–13.01, $n = 205$) and in the multivariate model was 4.43 (95% CI 1.09–18.01, $n = 190$) for each increasing OD unit. Twenty-seven (27%) of the cases had elevated adduct levels (tumor tissue) compared with 14 (13%) of the controls. Comparing the same samples, the odds ratio for elevated adducts was 2.40 (95% CI 1.18–4.92, $n = 205$). Controlling for common PAH exposure sources and known risk factors, elevated levels of PAH–DNA adducts were significantly associated with breast cancer case-control status (OR = 2.56, 95% CI 1.05 – 6.24, $n = 190$). Stratification by menopausal status did not significantly alter the odds ratio for elevated PAH–DNA adduct levels, but this analysis is limited by small numbers.

When cases and controls were compared using PAH–DNA adducts in nontumor tissue ($n = 90$) instead of tumor tissue ($n = 100$) from the cases, the presence of elevated adduct levels (21/90 cases versus 14/105) was associated with case-control status with borderline significance (OR 1.97, 95% CI 0.94 – 4.17, $n = 195$). Control for potential confounders and analyses using the continuous variable for adducts did not materially alter the odds ratio.

The benign breast disease control group had a higher prevalence of women with a family history of breast cancer than one might expect to find in a classic healthy control group, and this may have introduced a bias into the study. Indeed, controls with a family history had nonsignificantly higher adduct levels, with a geometric mean of 0.38 in women with a family history and 0.33 in women without a family history ($P = 0.26$). Although family history was controlled for in the main analyses, the effect of family history was further evaluated by reanalyzing the data in the subset of women without a family history, controlling for the other breast cancer risk factors and PAH exposure variables. Comparing adduct levels in tumor and benign tissues (OR=4.38, $P = 0.06$, multivariate model with adducts as a continuous variable) and nontumor and benign tissues (OR=1.94, $P = 0.47$, multivariate model with adducts as a continuous variable) the results in this subset were similar to those seen in the full dataset. Since a younger age at diagnosis is also associated with a genetic susceptibility to breast cancer, the data were

Table III. PAH–DNA adduct levels in breast tissue by demographic variables, tumor characteristics and exposure variables[a]

	Tumor tissue			Nontumor tissue			Benign tissue		
	Geometric mean	(GSD)	*n*	Geometric mean	(GSD)	*n*	Geometric mean	(GSD)	*n*
Ethnicity									
Caucasian	0.41	(1.88)	75	0.38	(1.65)	67	0.36	(1.62)	57
Non-Caucasian	0.33	(1.72)	25	0.34	(1.64)	23	0.32	(1.58)	48
Estrogen receptor status[b]									
Positive	0.36	(1.86)	59[c]						
Negative	0.26	(1.34)	13						
Menopausal status									
Pre-	0.38	(1.80)	34	0.32	(1.57)	33[c]	0.33	(1.60)	59
Post-	0.39	(1.88)	66	0.41	(1.69)	57	0.37	(1.60)	43
Smoking status									
Current	0.37	(2.08)	12	0.42	(1.92)	10	0.34	(1.86)	13
Ex	0.40	(1.82)	38	0.36	(1.65)	35	0.32	(1.54)	46
Never	0.38	(1.82)	49	0.37	(1.65)	44	0.36	(1.60)	45
Cigarettes smoked per day (during the past 2 years)									
0	0.39	(1.84)	82	0.37	(1.66)	75	0.35	(1.60)	82
>0–10	0.35	(1.40)	6	0.45	(1.31)	5	0.36	(1.57)	11
>10	0.35	(2.20)	10	0.38	(2.08)	8	0.28	(1.73)	10
Current ETS exposure in never smokers (during the past 2 years)									
Yes	0.35	(1.92)	17	0.35	(1.73)	15	0.30	(1.38)	15[c]
No	0.40	(1.79)	32	0.38	(1.63)	29	0.40	(1.65)	30
Current charred meat consumption (during the past year)									
High	0.30	(1.68)	30[c]	0.28	(1.37)	25[c]	0.30	(1.51)	36[c]
Low	0.43	(1.86)	68	0.41	(1.70)	63	0.36	(1.63)	65

[a]Values are expressed as the geometric mean (geometric standard deviation) in optical density units. *P* values were calculated by Student's *t*-test and ANOVA.
[b]Estrogen receptor data were not available for nontumor tissue from cases or benign tissue from controls.
[c]$P < 0.05$.

Table IV. Multiple linear regression analysis of possible predictors[a] of PAH–DNA adduct levels in breast tissue

	Slope (β) in tumor tissue ($n = 97$)	Slope (β) in nontumor tissue ($n = 87$)	Slope (β) in benign tissue ($n = 99$)
Ethnicity (Caucasian versus non-Caucasian)	0.02	0.03	0.09
Menopausal status (Post versus Pre)	0.03	0.20	0.156
Current active smoking (Yes versus no)	0.05	0.15	0.05
Current charred meat consumption (High versus low)	–0.30	–0.394[b]	–0.03
Current ETS exposure (Yes versus no)	–0.23	–0.05	–0.27[b]

[a]Each slope is adjusted for all other variables in the Table.
[b]$P < 0.05$, adjusted for other predictors.

also analyzed in the subgroup of women aged 40 years or older. After control for breast cancer risk factors and PAH exposure, the odds ratios for increasing adduct levels were similar to those seen in the full analyses. When adduct levels in tumor were compared with those in benign tissue, the odds ratio was 5.26 ($P = 0.03$, multivariate model with adducts as a continuous variable), and when nontumor was compared with benign tissue it was 1.98 ($P = 0.46$, multivariate model with adducts as a continuous variable).

Considering other possible environmental risk factors for breast cancer, the odds ratio for current smoking versus never smoking and former smoking was not significant (1.63, 95% CI 0.52 – 5.15), as was the odds ratio for current ETS exposure (1.31, 95% CI 0.60 – 2.89). However, the study had power only to detect odds ratios in the range of 2.8 for current smoking status and 2.2 for current ETS exposure.

Associations between adduct levels and ethnicity, estrogen receptor status, current active smoking, current ETS exposure, and consumption of charred food are presented in Tables III and IV. Adducts in tumor tissue were positively associated with the percentage of tumor cells staining positive for estrogen receptors ($\beta = 0.59$, $P = 0.01$). Overall, by multiple linear regression, active smoking did not significantly predict adducts. However, adducts in benign tissue were inversely related to current ETS exposure among the controls who had never actively smoked. In addition, high consumption of charred meat was inversely associated with adducts in nontumor tissue from cases. Overall, there was substantial (17-fold) variability in PAH–DNA adduct levels among the subjects.

Discussion

In this case-control study, PAH-DNA adducts measured in tumor tissue of breast cancer cases and in normal tissue from benign breast disease (BBD) controls showed a significant association with case-control status. When adducts in these tissues were considered on a continuous scale, after controlling for known risk factors and measured exposure sources, the odds ratio was 4.43 (95% CI 1.09–18.01) for each unit increase in optical density score. Considering adducts as a dichotomous variable, twice as many cancer cases as controls had elevated PAH–DNA adduct levels in breast tissue. The adducts in tumor and normal tissue from the cases were highly correlated ($P <$

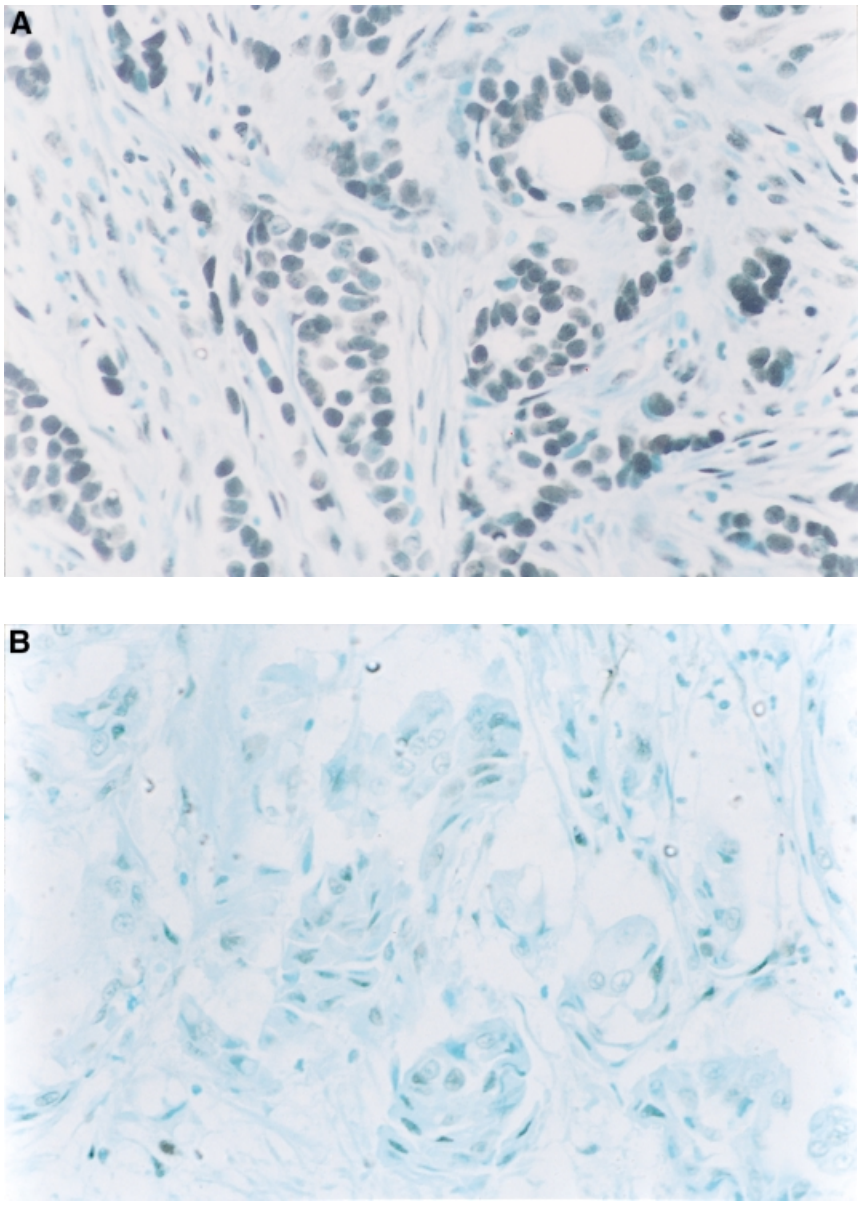

Fig. 2. (A) A tumor sample from a case with stage 1 breast cancer with intense nuclear staining with the anti-BPDE-DNA antibody. **(B)** A tumor sample from a case with stage 2 cancer with low-level staining with the anti-BPDE-DNA antibody. Both tissue sections are shown at 400× magnification.

0.001). However, while the relationship between adducts measured in nontumor tissue from cases and normal tissue from controls was in the same direction as that seen with tumor tissue, it was not statistically significant.

Two interpretations are possible. The first is that cases are inherently more sensitive to the carcinogenic effects of environmental PAH; and the conjunction of exposure and susceptibility played a causative role in their breast cancer. Supporting this interpretation is the fact that the cases were predominantly early stage, and adduct levels in paired tumor and nontumor tissue were significantly correlated. Additionally, although not statistically significant in part due to reduced numbers of nontumor ($n = 90$) versus tumor ($n = 100$), the odds ratio for adducts and case-control status, comparing nontumor tissue and benign tissue, was also elevated. A causative role of PAH is biologically plausible since experimental data show PAH to be potent mammary carcinogens in rodent bioassays (6,13) and because adducts are considered to be a critical step on the pathway to mutation and cancer (17,42,43), although their predictive value has not been established in prospective studies. There is considerable interindividual variability in PAH–DNA binding due to differences in PAH exposure as well as PAH activation/metabolism, DNA binding, and repair of PAH–DNA adducts (42,44,45). The finding reported here that, after controlling for two of the major PAH exposure sources (tobacco smoke and diet), PAH–DNA adduct levels in tumor tissue were positively associated with case-control status, might suggest that interindividual variation in metabolic and/or DNA repair pathways plays an important role in breast cancer (38).

However, a second possible interpretation is that progressive changes in the tumor cells lead to greater formation and accumulation of adducts in tumor tissue. Thus, the findings might have relevance to progression of tumors but not causation. It is possible that changes in metabolism or DNA repair and/or the increased availability of single-stranded DNA during

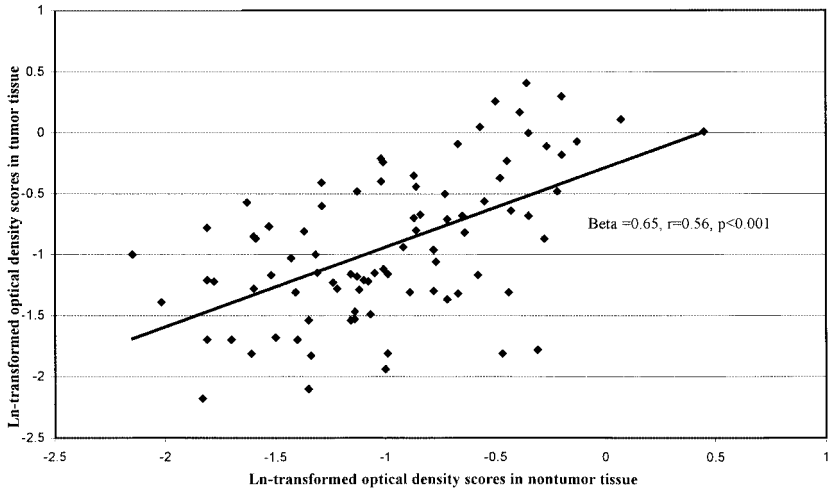

Fig. 3. Correlation analysis of adduct levels in paired tumor and nontumor tissue from cases. Levels expressed in natural log transformed optical density units.

replication and proliferation might lead to increased adduct levels. The finding of increased adduct levels in the tumor may be of biologic significance in that the accumulation of PAH–DNA could play a role in the further progression of the malignant cells. The increased concentration of adducts might lead over time to increased mutations and genomic instability, further contributing to the cancerous phenotype of the cells.

A limitation of the study was our inability to determine the specific contributions of all of the possible PAH exposure sources, both past and present, to the PAH–DNA adducts measured in breast tissue. The adduct measurements are able to integrate a multiplicity of exposures, including tobacco smoke, dietary PAH, indoor and outdoor air pollution, but our questionnaire included only tobacco smoke and dietary exposure. In addition, although the lifetime of adducts in human breast tissue is not known, PAH are fat soluble and can accumulate in adipose tissue to be released over time and redistributed to the breast epithelial cells (46). Thus, adducts may reflect long-term as well as recent exposure. Although some data on lifetime environmental exposures were elicited by the questionnaire, the analysis of exposure–adduct relationships relied largely on current measures due to their greater reliability. Our analyses have not included nutritional and genetic factors and consideration of possible gene–environmental interactions that might help explain the variability in individual levels of adducts in breast tissue and breast cancer risk (47–50). In addition, the finding that adduct levels are associated with the expression of estrogen receptors suggests that hormone signaling, perhaps by influencing cell turnover, may affect adduct formation. Thus, intra- and inter-individual differences in hormone levels may create variability in adduct levels that obscures associations with exposure variables (51). The observations that active and passive smoking were not associated with adducts overall but that there were significant inverse relationships between ETS and adducts (benign tissue only) and charred meat (nontumor tissue from cases only) should be viewed in this context and are limited accordingly.

This study was designed to address the major limitations of prior studies of PAH– and aromatic–DNA adduct levels in breast tissue, which were primarily the inappropriate use of reduction mammoplasty controls and the failure to consider possible confounders. In this type of study, the choice of control subjects is necessarily constrained by the need to collect breast tissue samples for adduct analysis, while drawing controls from the population of healthy women, who if they had breast-related complaints, would have come to the same hospital from which the cases were drawn. Here, women with benign breast disease can be considered to be representative of this population since they came to the hospital for breast-related complaints through the same referral routes as the cases. In contrast, women seeking reduction mammoplasties are clearly not representative of this population, having larger breasts, and probably being younger and of higher socioeconomic class. Further, reduction mammoplasties are not performed by the Breast Service, but by the Division of Plastic and Reconstructive Surgery; and it is not clear whether the referral patterns of these two services overlap.

Another concern is that the use of benign breast disease controls may have biased the study results towards the null. While the elevated risk of future breast cancer after a diagnosis of a benign condition is predominantly found among women with atypia (who were excluded from this study), benign breast disease patients without atypia have a slightly elevated risk of future breast cancer (52). Thus, our controls may have shared more risk factors (both measured and unmeasured) with the cases than a control group of healthy women drawn from the source population. If these risk factors were associated with adduct levels, there would be increased homogeneity among our study subjects, biasing our adduct odds ratio estimates towards the null. Such an effect was seen in a similarly designed breast cancer case-control study that drew one healthy control group from a breast referral clinic and a second from general medical clinics (53). To evaluate possible confounding factors and to demonstrate that the control group was appropriate, a logistic regression analysis of known breast cancer risk factors was performed. The results were consistent with the literature in that age, ethnicity and current regular alcohol consumption were significantly associated with breast cancer case-control status.

Family history of breast cancer was not positively associated with case-control status due to a high prevalence of women with a family history among the BBD controls. As suggested by past studies of women with benign breast disease, the increased prevalence probably reflects greater medical surveillance among women with a family history and probably a lower threshold for surgical referral for such women (54–56).

Bias would have been introduced into the study if a family history were associated with increased adduct levels, and indeed, controls with a family history of breast cancer had nonsignificantly higher adduct levels, so it is possible that a bias to the null was introduced. However, family history of breast cancer was controlled for in the multivariate models, which should have removed this possible bias. Further, the subset analyses of women without a family history of breast cancer and women older than age 40 found similar associations as the full analysis, indicating that any potential bias was probably adequately controlled for in the full multivariate model.

A limitation of the immunohistochemical assay is that results cannot be reported in adduct units; e.g. as adducts per 10^8 nucleotides (34). Although the antibody was raised against BPDE–DNA adducts, it crossreacts with other PAH that form diol epoxide adducts, and does so with varying affinities (34,57,58). Humans are exposed to complex mixtures of PAH, and multiple adduct species are likely to be present. Since the composition of this mixture cannot be predicted and probably varies from individual to individual, relevant standards derived from treated cell lines or animals cannot readily be made. In addition, the antibody may cross-react with other substances of unknown structures. However, immunohistochemical assays for PAH–DNA adducts, including several that have used the same antibody (5D11), have previously demonstrated the ability to reflect differences in environmental exposures to PAH as a class of chemicals (19,21,23,24,26,30,31,59). This study reports on PAH–DNA adduct levels in breast tissue from a large series of cases and controls drawn from and representative of the same parent population. It is the first to investigate the possible association between PAH–DNA adduct levels in tumor and nontumor tissue and breast cancer status, controlling for other risk factors related to breast cancer. Other advantages of the study include the fact that tissues were obtained prior to any treatment and the relatively large numbers of subjects with biomarker and questionnaire data.

Our results suggest that genetic damage from environmental sources of PAH may be a risk factor for breast cancer causation or progression. However, additional studies are needed of larger numbers of nontumor samples from cases to elucidate the role of these environmental contaminants.

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