Null Results in Brief

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Breast Cancer and Urinary Biomarkers of Polycyclic Aromatic Hydrocarbon and Oxidative Stress in the Shanghai Women's Health Study

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Abstract

Polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress from such and other exposures have been associated with breast cancer in some studies. To further evaluate the role of PAH metabolites and oxidative stress on the development of breast cancer, we conducted a nested case-control study in the Shanghai Women's Health Study.

We measured urinary 1-hydroxypyrene and 2-naphthol as PAH metabolites and urinary levels of 8-hydroxy-2′-deoxyguanosine and malondialdehyde as oxidative stress biomarkers in 327 breast cancer cases and 654 controls in the Shanghai Women's Health Study. Information on demographic characteristics, past medical history, lifestyles, history of menstruation, pregnancy history, eating and drinking habit, history of residence, employment history, family history, husband's information, and physical activity were collected by a self-administered questionnaire.

The mean age was 52.3 in breast cancer cases (n = 354) and 52.5 in controls (n = 708). Age at menarche (P = 0.04), months of breast-feeding the first baby (P = 0.05), and grade of education (P_{trend} < 0.01) were significantly different between cases and controls. No association was observed for PAH metabolites and the oxidative stress biomarkers of urinary malondialdehyde and 8-hydroxy-2′-deoxyguanosine and risk of breast cancer.

This nested case-control study provides no evidence of association between PAH exposure and oxidative stress and risk of breast cancer in Shanghai women. *Cancer Epidemiol Biomarkers Prev; 19(3); 877–83. ©2010 AACR.*

Introduction

Well-established risk factors for breast cancer (early age at menarche, late age of menopause, late age at first pregnancy, obesity, use of oral contraceptives and hormone replacement therapy, diet, family history, lactation, and prior history of benign breast disease) do not fully explain the occurrence of the disease (1, 2). Some environmental factors such as air pollutants, pesticides, and polycyclic aromatic hydrocarbons (PAH) have been suggested to play a role in the pathogenesis of breast cancer, but evidence is inconclusive (3). PAHs have been shown to accumulate in breast tissue (4) and lead to breast cancer in humans (5, 6). A potential limitation of most epidemiologic studies of breast cancer and PAHs is that information on exposure was obtained by questionnaire rather than ambient air or biological measurements. PAH metabolites, including 1-hydroxypyrene (1-OHP) and 2-naphthol, have been directly measured from human specimens in various studies of occupational PAH exposure (7), nonoccupational exposure (8), air pollution (9), and cigarette smoke (10).

A postulated carcinogenic pathway for PAHs and other chemicals, or their metabolites, involves the production of reactive oxygen species, which causes oxidative stress and can lead to lipid peroxidation, protein modification, membrane disruption, and mitochondrial and DNA damage (11). This postulated pathway can be evaluated using malondialdehyde (MDA) as an indicator for oxidative stress and lipid peroxidation (12) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) as an indicator for DNA damage. 8-OHdG is the most abundant DNA lesion caused by reactive oxygen species; after cleavage from DNA during DNA repair, it is excreted in urine (13) and can serve as a general biomarker of oxidative stress. Increased levels of 8-OHdG have been observed among patients with small cell

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lung, bladder, and prostate cancers and may be a potential risk factor for breast cancer (13). Serum levels of total antioxidant capacity and lipid peroxidation products, such as MDA and lipid hydroperoxide, were used as markers of oxidative stress in a breast cancer study (14), which found MDA levels among breast cancer patients higher than among controls (14). Other small studies have also reported increased levels of 8-OHdG in peripheral lymphocytes and MDA in plasma of breast cancer patients relative to controls (15, 16). Oxidative stress can also cause mutations of tumor suppressor genes that are critical initial events in breast carcinogenesis (17). Although changes in serum levels of MDA have been related to breast cancer (14), the role of lipid peroxidation was controversial.

We conducted a nested case-control study in the Shanghai Women's Health Study (SWHS; ref. 18), a population-based prospective cohort, to further evaluate the role of PAH exposure and oxidative DNA damage in the development of breast cancer.

Materials and Methods

Study Population

The SWHS provided data and biological specimens for a nested case-control study of 354 breast cancer cases and 708 controls. Breast cancer cases were identified by active contact with cohort members and through the Shanghai Tumor Registry between enrollment (1997-2000) and the end of 2004. Urine samples for 327 of the 354 cases were available for analysis of biological markers.

Controls were selected from SWHS participants alive and free from cancer at the time the case was diagnosed. Two controls were matched to each case by age at baseline (±2 years), sample collection date (<31 days), biological specimen collection time (A.M. or P.M.), antibiotic use in the past week (yes/no), and menopause status (for postmenopausal women, the same menopausal status on the date of urine collection; for premenopausal women, difference of last period date and sample collection date ≤3 days). Of the 708 eligible controls identified, urine samples were not available for 54 and urinary biomarkers were analyzed for 654. The study was approved by the relevant institutional review boards for human research at the Shanghai Cancer Institute in China and the National Cancer Institute in the United States. All participants provided informed written consent.

Questionnaire and Biospecimen Collection

At cohort enrollment, participants provided information on demographics, dietary intake, disease history, personal habits (e.g., alcohol and tobacco consumption, environmental exposure to tobacco smoke, tea drinking, and hair dye use), menstrual and reproductive history, residential history, occupational history, family history of cancer, and physical activity. A spot urine sample was collected in containers spiked with ascorbic acid (100 mg/100 mL urine) to prevent oxidation of labile metabolites. After collection, the samples were kept at 4°C until transported to the Shanghai Cancer Institute Central Laboratory for processing. All specimens were processed within 6 h of collection and stored at −70°C. Urine samples are available on ~88% of the cohort participants.

Materials

Reference standards of 1-OHP and 2-naphthol standard, 2-thiobarbituric acid, and MDA standard were obtained from Sigma-Aldrich. Hydrolysis reagent β-glucuronidase/arylsulfatase was from Roche Molecular Biochemicals. Methanol and acetonitrile with a purity of 99.85% were from Hayman. All other chemicals were obtained in the greatest purity available from commercial suppliers. The commercial ELISA kit for 8-OHdG, designed for quantitative measurement of the oxidative DNA adducts 8-OHdG in urine, was from the Japan Institute for the Control of Aging.

Assay of Urinary 1-OHP

Urinary 1-OHP was determined using reverse-phase high-performance liquid chromatography (HPLC; ref. 19). In brief, 0.5 mL urine samples were buffered with 50 μL of 2.0 mol/L sodium acetate buffer (pH 5.0) and hydrolyzed with 10 μL β-glucuronidase/sulfatase (Sigma). The urine mixture was incubated at 37°C for 16 h in a shaking water bath. After hydrolysis, 0.5 mL acetonitrile was added to the mixture. The mixture was centrifuged and 100 μL of the supernatant were taken for HPLC application. HPLC system was constituted with Young-Lin SP930D HPLC Pump, Young-Lin Automated Gradient Controller, *MIDAS* 830 Autosampler, and *JASCO* FP-2020 Plus Fluorescence Detector. The following HPLC parameters were used: column, Sunfire C_{18} (4.5 × 250 mm); mobile phase, 50% acetonitrile in water; flow rate, 0.8 mL/min. Excitation/emission wavelength used in the detection of 1-OHP was 277/388 nm. The limit of detection of 1-OHP was 0.1 ng/mL. The recovery of the assay was 82%, and the coefficient of variation of the assay was 9%. Standard curve had correlation coefficient >0.99 and showed good linearity.

Analysis of Urinary 2-Naphthol

Urinary 2-naphthol was determined using reverse-phase HPLC. In brief, 0.5 mL urine samples were buffered with 50 μL of 2.0 mol/L sodium acetate buffer (pH 5.0) and hydrolyzed with 10 μL β-glucuronidase/sulfatase (Sigma). The urine mixture was incubated at 37°C for 16 h in a shaking water bath. After hydrolysis, 0.5 mL acetonitrile was added to the mixture. The mixture was centrifuged and 100 μL of the supernatant were taken for HPLC application. HPLC system was constituted with Young-Lin SP930D HPLC Pump, Young-Lin Automated Gradient Controller, *MIDAS* 830 Autosampler, and *JASCO* FP-2020 Plus Fluorescence Detector. The following HPLC parameters were used: column, Sunfire C_{18} (4.5 × 250 mm); mobile phase, 50% acetonitrile in water;

flow rate, 0.8 mL/min. Excitation/emission wavelength used in the detection of 2-naphthol was 227/355 nm. The limit of detection was 0.5 ng/mL, and the coefficient of variation was <15% (20).

Analysis of Urinary MDA

The most common method of measuring MDA is based on the reaction with 2-thiobarbituric acid. A 10 mmol/L stock standard of MDA was prepared by dissolving 123.5 μL 1,1,3,3-tetraethoxypropane in 50 mL ethanol (40% ethanol by volume). 2-Thiobarbituric acid-MDA adducts were prepared in glass tubes with a polypropylene stopper. In each tube, 300 μL phosphoric acid (0.5 mol/L) was mixed with 50 μL urine and 150 μL 2-thiobarbituric acid reagent. The mixtures were incubated at 95°C for 1 h, and methanol (500 μL) was added in each tube. After 5 min centrifugation (5,000 × *g*), the samples were analyzed using HPLC on a 4.6 × 150 mm Sunfire C_{18} column with UV detector with 532 nm wavelength. The mobile phase was potassium phosphate (0.05 mol/L; pH 6.8) and methanol (58:42, v/v). The flow rate was 0.8 mL/min. MDA (Sigma-Aldrich; T-8998) was used as an external standard. MDA standards (0.1, 0.5, 1, 2, and 4 μmol/L) were prepared with 1,1,3,3-tetramethoxypropane. The limit of detection was 0.05 μmol/L, the correlation for the linearity of the standard curve was 0.99, and the coefficient of variation was <10% (20).

Analysis of Urinary 8-OHdG

The level of urinary 8-OHdG was determined by a competitive ELISA kit (Japan Institute for the Control of Aging). In brief, 50 μL primary monoclonal antibody and 50 μL sample or standard were added to microliter plates, which were precoated with 8-OHdG, incubated at 37°C for 1 h, and washed with 250 μL PBS. Horseradish peroxidase–conjugated secondary antibody (100 μL) was then added to each well, incubated at 37°C for 1 h, and washed with 250 μL PBS. Enzyme substrate (100 μL) was then added to each well and incubated at 37°C for 1 h, and the reaction was terminated with 100 μL of 1 N phosphoric acid. Absorbance of each well was read at 450 nm by a microplate reader (ELx808; Bio-Tek). The 8-OHdG concentration of the urine samples was interpolated from a standard curve drawn with the assistance of logarithmic transformation. The coefficient of variation of the assay was within 12% during the period of sample analysis. The limit of detection was 0.5 ng/mL (21).

Statistical Methods

Log-transformed data were used for the statistical analysis after confirming that the urinary levels of biomarkers were approximately log-normally distributed. The biomarker data were expressed as geometric mean ± geometric SD. Differences between case-control pairs in mean concentrations of PAH metabolites and oxidative stress biomarkers were tested with the use of a paired *t* test for matched data, and the χ^2 test was used for categorical variables. All tests of significance were two-sided. The criterion for significance was set at $P < 0.05$.

We examined the association between urinary biomarkers and breast cancer risk in conditional logistic regression model adjusting for potential confounding factors. The adjusted odds ratio (OR) and exact computation of 95% confidence intervals (95% CI) were calculated. Cases and controls were categorized according to the quartile distribution of urinary excretion levels of biomarkers among the controls. To evaluate the potential confounding factors, known breast cancer risk factors in this population and characteristics of study participants were compared between cases and controls. All statistical analyses were done with SPSS Statistical Package version 12.0 (SPSS).

Results

Cases and controls were comparable in age at interview (mean age, 52.3 and 52.5, respectively), as they were matched by age (Table 1). Compared with controls, cases were younger at menarche ($P = 0.08$, χ^2 test) and older at menopause ($P_{trend} = 0.02$). An earlier age at first birth and increased number of births reduced the risk of breast cancer ($P_{trend} < 0.01$ and $P_{trend} = 0.05$, respectively; Table 1). Significant differences between cases and controls included months of breast-feeding the first baby (OR, 0.75; 95% CI, 0.56-0.99) and education (OR, 0.40; 95% CI, 0.25-0.64 for college educated; Table 1). Although not statistically significant, breast cancer risk tended to be lower among those with later menarche (OR, 0.79; 95% CI, 0.61-1.02) and those ever pregnant (OR, 0.76; 95% CI, 0.37-1.55; Table 1).

Urinary levels of the biomarkers examined, with or without adjustment for creatinine, were not significantly different between breast cancer cases and controls (Table 2). The unadjusted geometric means of urinary 1-OHP were 1.9 ng/mL for cases and 2.0 ng/mL for controls ($P = 0.09$, paired *t* test), 4.4 and 4.9 ng/mL for 2-naphthol, 2.0 and 2.1 μmol/L for MDA, and 9.7 and 9.8 ng/mL for 8-OHdG, respectively (Table 2). Adjustment for creatinine altered the values of all of the markers similarly for cases and controls and did not lead to any significant differences.

The risk of breast cancer was not significantly associated with urinary levels of 1-OHP and 2-naphthol (Table 3). Likewise urinary MDA and 8-OHdG, the oxidative stress biomarkers, were not significantly associated with a risk of breast cancer (Table 3). We also evaluated the relationship between urinary biomarkers and risk of breast cancer stratified by menopause status and found no significant associations (data not shown).

Discussion

Results from this nested case-control study among participants in the SWHS did not support an association between PAH metabolites, oxidative stress, and risk of breast cancer. Statistical powers ranged from 43% to 79% (43% for 8-OHdG and 79% for 1-OHP). We found

Table 1. Characteristics of cases and their matched control subjects from the SWHS

	Cases		Controls		OR (95% CI)	P*
	n	%	*n*	%		
Age at interview (y; mean ± SE)	354	52.3 ± 0.5	708	52.5 ± 0.3		0.81
Age at menarche (y)						
<15	165	46.6	289	40.9	1	
≥15	189	53.4	418	59.1	0.79 (0.61-1.02)	0.08
Age of menopause (y)						
<46	24	14.0	62	17.9	1	
46-49	35	20.3	86	24.9	1.05 (0.57-1.94)	
49-50	22	12.8	60	17.3	0.95 (0.48-1.87)	
≥50	91	52.9	138	39.9	1.70 (0.99-2.92)	0.02
Menopausal status						
Premenopausal	182	51.4	361	51.1	1	
Postmenopausal	172	48.6	346	48.9	0.98 (0.76-1.27)	0.99
Body mass index						
<23.4	163	50.3	311	50.1	1	
≥23.4	161	49.7	310	49.9	0.99 (0.76-1.30)	0.95
Age at first birth (y)						
<24	69	20.2	185	26.7	1	
24-27	81	23.8	181	26.2	1.20 (0.82-1.75)	
27-29	82	24.0	145	21.0	1.52 (1.03-2.23)	
≥29	109	32.0	181	26.2	1.61 (1.12-2.32)	<0.01
No. births						
<2	76	21.5	122	17.1	1	
2-3	109	30.8	212	29.8	0.83 (0.57-1.19)	
≥3	169	47.7	378	53.1	0.72 (0.51-1.01)	0.05
Breast-feeding the first baby (mo)						
<10	174	58.2	308	51.1	1	
≥10	125	41.8	295	48.9	0.75 (0.56-0.99)	0.04
Education						
Elementary or less	61	17.3	78	11.0	1	
Junior high school	124	35.1	192	27.1	0.82 (0.55-1.24)	
High school	119	33.7	282	39.8	0.54 (0.36-0.80)	
College or more	49	13.9	156	22.0	0.40 (0.25-0.64)	<0.01
Family history of disease (tumor or cancer)						
No	247	69.8	524	74.0	1	
Yes	107	30.2	184	26.0	1.23 (0.93-1.64)	0.14
Environmental tobacco smoke (home and/or work)						
No	78	24.0	138	20.6	1	
Yes	247	76.0	533	79.4	0.82 (0.60-1.13)	0.22
Fried fish, meat, chicken, and duck intake						
No	110	31.1	183	25.8	1	
Yes	244	68.9	525	74.2	0.77 (0.58-1.02)	0.07
Drinking tea (green/block/oolong/scented)						
No	243	68.6	496	69.7	1	
Yes	111	31.4	216	30.3	1.05 (0.80-1.38)	0.73

NOTE: Matching variables: age of baseline ±2 years, sample collection date <31 days + A.M./P.M. match, antibiotic use in the past week, previous cancer history, and menopausal status (for postmenopausal, the same menopausal status on the data in collected urine; for premenopausal, difference of last period data and sample collection data ≤3 days).

*By Student's *t* test (continuous variables); P_{trend} or χ^2 test (categorical variables).

Table 2. Levels of urinary biomarkers of cases and their matched control subjects from the SWHS

	Cases		Controls		*P**
	n	Geometric mean ± SD	*n*	Geometric mean ± SD	
1-OHP (ng/mL)	327	1.9 ± 2.3	654	2.3 ± 1.8	0.12
2-Naphthol (ng/mL)	327	4.3 ± 2.3	654	5.2 ± 1.9	0.34
MDA (μmol/L)	327	2.0 ± 2.2	654	2.3 ± 1.7	0.27
8-OHdG (ng/mL)	327	8.3 ± 2.3	654	9.0 ± 1.9	0.31
Adjusted for creatinine					
1-OHP (μmol/mol creatinine)	326	1.6 ± 2.2	652	1.7 ± 2.0	0.76
2-Naphthol (μmol/mol creatinine)	326	5.6 ± 2.3	652	6.6 ± 1.9	0.49
MDA (mg/g creatinine)	326	0.7 ± 2.1	652	0.7 ± 1.7	0.85
8-OHdG (μg/g creatinine)	326	11.9 ± 1.6	652	11.8 ± 1.7	0.43

*By paired *t* test.

that several well-established risk factors were also important predictors of breast cancer in our study, including age at menarche, age at menopause, age at first birth, number of births, and duration of breast-feeding the first baby (1). These observations verified the differences in risk profile between breast cancer cases and controls in our study and supported our use of these data for further exploration of environmental risk factors.

PAHs have been associated with lung, bladder, and breast cancers in animals (22) and in humans (23). PAH-DNA adducts on a natural scale were slightly, but nonsignificantly, higher among breast cancer cases than among controls in the Long Island Breast Cancer Study Project (1996-1997; ref. 24). However, in our study, urinary 1-OHP and 2-naphthol levels were higher among controls, although none of the differences were statistically

Table 3. Adjusted OR (95% CI) for breast cancer associated with urinary 1-OHP, 2-naphthol, MDA, and 8-OHdG

Risk factor	Cases (%)	Controls (%)	Adjusted OR (95% CI)	P_{trend}
1-OHP (μmol/mol creatinine)				
Q1: ≤1.018	92 (26.8)	159 (25.0)	1	
Q2: >1.018 to ≤1.563	84 (24.5)	160 (25.1)	0.90 (0.62-1.30)	
Q3: >1.563 to ≤2.452	81 (23.6)	159 (25.0)	0.83 (0.57-1.20)	
Q4: >2.452	86 (25.1)	159 (25.0)	0.91 (0.63-1.32)	0.54
2-Naphthol (μmol/mol creatinine)				
Q1: ≤3.901	96 (28.0)	159 (25.0)	1	
Q2: >3.901 to ≤6.162	87 (25.4)	160 (25.1)	0.93 (0.64-1.35)	
Q3: >6.162 to ≤10.602	81 (23.6)	159 (25.0)	0.81 (0.56-1.18)	
Q4: >10.602	79 (23.0)	159 (25.0)	0.83 (0.58-1.21)	0.26
MDA (mg/g creatinine)				
Q1: ≤0.481	86 (25.1)	156 (24.5)	1	
Q2: >0.481 to ≤0.677	86 (25.1)	162 (25.4)	0.96 (0.66-1.39)	
Q3: >0.677 to ≤1.011	85 (24.8)	160 (25.1)	0.96 (0.66-1.40)	
Q4: >1.011	86 (25.1)	159 (25.0)	0.98 (0.67-1.42)	0.91
8-OHdG (μg/g creatinine)				
Q1: ≤10.364	79 (23.0)	166 (26.1)	1	
Q2: >10.364 to ≤14.497	93 (27.1)	152 (23.9)	1.28 (0.88-1.86)	
Q3: >14.497 to ≤20.209	86 (25.1)	159 (25.0)	1.13 (0.78-1.65)	
Q4: >20.209	85 (24.8)	160 (25.1)	1.11 (0.76-1.62)	0.77

NOTE: Matching variables: age of baseline ±2 years, sample collection date <31 days + A.M./P.M. match, antibiotic use in the past week, previous cancer history, and menopausal status (for postmenopausal, the same menopausal status on the data in collected urine; for premenopausal, difference of last period data and sample collection data ≤3 days).

significant. PAH exposures could have been from lifestyle factors such as environmental tobacco smoke and eating fried fish and meat than cases, as shown in Table 1, or occupational factors (5). We have found previously that environmental tobacco smoke and grilled fish (or shell) consumption were important predictors of urinary PAH metabolite in South Korean children ($n = 102$; ref. 8).

We did not find a significant association between urinary 8-OHdG levels and risk of breast cancer, in contrast to previous studies (25, 26). In recent years, using MDA as a biomarker of oxidative stress, there has been a growing interest in a possible role for lipid peroxidation in cancer development. Elevated levels of lipid peroxidation products have been detected in breast cancer patients and in women at high risk for breast cancer as opposed to controls (27, 28). Urinary F2-isoprostanes, products of lipid peroxidation, were positively associated with breast cancer risk among overweight women (25). On the other hand, some literature suggests that lipid peroxidation product might protect against breast cancer (26). Studies of rodents and cultured breast cancer cells also suggest that increased cytotoxic lipid peroxidation products may play a role in breast cancer protection (29). Gago-Dominguez et al. provided supportive evidence in humans by implicating the peroxidation products of marine *n*-3 fatty acids as the proximal anticarcinogens (30). Although estrogen metabolism may serve as a source of oxidative stress (31), estrogens, known to increase breast cancer risk, have been found to inhibit lipoprotein peroxidation *in vivo* and *in vitro* (32). In this study, the risk of breast cancer was not associated with urinary levels of lipid peroxidation products as MDA.

This study had several strengths. A major strength is that urine samples and risk factor information were collected before diagnosis of breast cancer, minimizing the possibility of recall bias on questionnaire data, or influence of the disease process on biological measurements. Detailed information was available on established and potential confounders, and adjustments were possible in the analysis. There are some limitations. Urines were spot samples. Although spot urine sample results were more practical, there are problems with interpreting the urinary creatinine levels that are used to gauge the correction to a $\geq$24 h urine output.

This nested case-control study did not show an association between PAH metabolites and oxidative stress markers and risk of breast cancer. Our findings suggest that PAH exposure may not be an important risk factor for breast cancer, and urinary PAH metabolites reflect short-term carcinogen exposures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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