Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

Cancer Epidemiology, Biomarkers & Prevention

Research Article

Elevated 4-Aminobiphenyl and 2,6-Dimethylaniline Hemoglobin Adducts and Increased Risk of Bladder Cancer among Lifelong Nonsmokers—The Shanghai Bladder Cancer Study

Li Tao[1], Billy W. Day[2], Bibin Hu[2], Yong-Bing Xiang[5], Renwei Wang[4], Mariana C. Stern[6], Manuela Gago-Dominguez[6,7], Victoria K. Cortessis[6], David V. Conti[6], David Van Den Berg[6], Malcolm C. Pike[6,8], Yu-Tang Gao[5], Mimi C. Yu[6], and Jian-Min Yuan[4,3]

Abstract

Background: 4-Aminobiphenyl (ABP) is an established human bladder carcinogen, with tobacco smoke being a major source of human exposure. Other arylamine compounds, including 2,6-dimethylaniline (2,6-DMA), have been implicated as possible human bladder carcinogens. Hemoglobin adducts of 4-ABP and 2,6-DMA are validated biomarkers of exposure to those compounds in humans.

Methods: The Shanghai Bladder Cancer Study enrolled 581 incident bladder cancer cases and 604 population controls. Each participant was solicited for his/her history of tobacco use and other lifestyle factors and donation of blood and urine specimens. Red blood cell lysates were used to quantify both hemoglobin adducts of 4-ABP and 2,6-DMA. Urine samples were used to quantify total cotinine. ORs and 95% confidence intervals (CI) for bladder cancer were estimated using unconditional logistic regression methods.

Results: Among lifelong nonsmokers, ORs (95% CIs) of bladder cancer for low (below median of positive values) and high versus undetectable levels of 2,6-DMA hemoglobin adducts were 3.87 (1.39–10.75) and 6.90 (3.17–15.02), respectively ($P_{trend} < 0.001$). Similarly, among lifelong nonsmokers, ORs (95% CIs) of bladder cancer for third and fourth versus first/second quartiles of 4-ABP hemoglobin adducts was 1.30 (0.76–2.22) and 2.29 (1.23–4.24), respectively ($P_{trend} = 0.009$). The two associations were independent of each other.

Conclusion: Hemoglobin adducts of 4-ABP and 2,6-DMA were significantly and independently associated with increased bladder cancer risk among lifelong nonsmokers in Shanghai, China.

Impact: The findings of the present study in China with previous data in Los Angeles, California strongly implicate arylamines as potential causal agents of human bladder cancer. *Cancer Epidemiol Biomarkers Prev; 22(5); 937–45. ©2013 AACR.*

Introduction

Early observational studies show significantly increased incidence of bladder cancer among workers occupationally exposed to arylamines in the workplace, and subsequent studies led to the discovery and confirmation of certain arylamines, including 4-aminobiphenyl (4-ABP), 2-naphthylamine, and benzidine, to be carcinogenic to humans. They are classified as group I carcinogens by the International Agency for Research on Cancer (IARC; ref. 1).

Following governmental regulation of industrial use of arylamines in 1970s, tobacco smoke emerged as the major source of 4-ABP exposure in humans (2). Tobacco use is recognized as an important risk factor for bladder cancer. Smokers exhibit 2- to 3-fold of excess risk for bladder cancer compared with nonsmokers (3). In our case–control study in Los Angeles, California, smokers exhibit more than 3 times higher levels of 4-ABP hemoglobin adducts, a validated biomarker of 4-ABP exposure, than lifelong nonsmokers (4).

Although tobacco use is a known major risk factor for bladder cancer, it only accounts for roughly 50% of disease burden in the United States (5). Relatively little is known about the causes of nonactive smoking related bladder

Authors' Affiliations: [1]Cancer Prevention Institute of California, Fremont, California; [2]Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh; [3]Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh; [4]Division of Cancer Control and Population Sciences, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania; [5]Department of Epidemiology, Shanghai Cancer Institute and Cancer Institute of Shanghai Jiaotong University, Shanghai, China; [6]Department of Preventive Medicine, Keck School of Medicine, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California; [7]Galician Foundation of Genomic Medicine, Complexo Hospitalario Universitario Santiago (CHUS), Servicio Galego de Saude (SERGAS), Instituto de Investigacion Sanitaria Santiago (IDIS), Santiago de Compostela, Spain; and [8]Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York

Corresponding Author: Jian-Min Yuan, University of Pittsburgh Cancer Institute, 5150 Central Avenue, Pittsburgh, PA 15232. Phone: 412-864-7889; Fax: 412-623-3303; E-mail: yuanj@upmc.edu

doi: 10.1158/1055-9965.EPI-12-1447

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

cancer. We have reported environmental tobacco smoke (ETS) exposure as a risk factor for nonsmoking-related bladder cancer (6, 7). The data were based on 2 separate but parallel case–control studies conducted in Los Angeles, California (high-risk region; The Los Angeles Bladder Cancer Study) and Shanghai, China (low-risk region; The Shanghai Bladder Cancer Study). The Los Angeles Study further implicates other diffuse sources of 4-ABP (besides active and passive smoking) as possible causal factors for nonsmoking-related bladder cancer.

More significantly, The Los Angeles Study examined hemoglobin adducts of nine alkylanilines, a subclass of arylamines that was previously unstudied in relation to bladder cancer risk, and found 3 of them, including 2,6-dimethylaniline (2,6-DMA), to be independently and significantly associated with bladder cancer risk among nonsmoking subjects at blood draw. Experimental studies have shown that administration of 2,6-DMA in the diet can induce adenomas and carcinomas as well as several sarcomas in the nasal cavity of rats. 2,6-DMA also produces subcutaneous fibromas and fibrosarcomas and increases the incidence of neoplastic nodules in the livers of rats (8, 9). 2,6-DMA is classified by IARC as a group 2B carcinogen (possibly carcinogenic to humans; refs. 9, 10).

Arylamines require metabolic activation, usually in the liver, to be transformed into fully carcinogenic agents (11). They are oxidized to *N*-hydroxylamines *in vivo* and react with hemoglobin to form adducts that may persist for as long as the hemoglobin adducts remains in circulation (4, 12). In the acidic environment of the bladder lumen, a derivative of the glucuronide conjugates of *N*-hydroxylamines can covalently bind to urothelial DNA and cause malignant transformation of urothelial cells, which may ultimately lead to bladder cancer (13). Hemoglobin adducts of arylamines are validated biomarkers of arylamine exposure in humans (14). These adducts represent both the uptake of arylamines and their genetically controlled metabolism in individual subjects.

Here, we report the findings of the Shanghai Bladder Cancer Study on hemoglobin adducts of 4-ABP and 2,6-DMA in relation to bladder cancer risk. The results are consistent with our prior novel findings in Los Angeles (4, 12) and strongly implicate arylamines as major causal agents of bladder cancer, not only among tobacco users but in lifelong nonsmokers as well.

Materials and Methods

Subjects

The present study included participants of the Shanghai Bladder Cancer Study. The study design has been described in detail elsewhere (7). Briefly, patients with bladder cancer were identified through the Shanghai Cancer Registry, a population-based cancer registry covering the approximately 8 million residents of urban area in Shanghai, China, in the 1990s. Of the patients diagnosed with bladder cancer from July 1, 1995, to June 30, 1998, 708 were 25 to 74 year old who met our eligibility criteria for the study. Among the 708 patients, 56 died before we could contact them, 29 refused to be interviewed, and 42 were unable to be located. We interviewed the remaining 581 (82%) eligible patients between July 1996 and June 1999. The diagnosis of bladder cancer for 531 (91%) patients was made based on histopathologic evidence, whereas the remaining 50 (9%) patients diagnoses were based on positive computerized axial tomography scan and/or ultrasonograph with consistent clinical history. Control subjects were randomly selected from the urban population of Shanghai through the Residents Registry of the Shanghai Municipal Government. They were chosen to match the frequency distribution by sex and 5-year age groups of patients with bladder cancer. Among the 750 potential control subjects chosen, 604 (81%) eligible subjects were interviewed during the same time period as the cases. All subjects provided informed consent following procedures approved by the appropriate institutional review boards.

Data collection

A trained interviewer conducted an in-person interview with each study subject using a structured questionnaire. The questionnaire asked for information on subjects' demographic characteristics, history of tobacco use, history of passive smoking (for nonsmokers only), consumption of beverages, use of hormones (for women only), medical history, usual adult diet, and occupational history. Cigarette smoking was defined as smoking 1 cigarette per day for at least 6 months. Lifelong nonsmokers were defined as subjects who were not smoking on a regular basis in the past 6 months before the reference date (2 years before cancer diagnosis for cases and 2 years before interview for controls) and who did not smoke any cigarettes in the past 6 months prior to the date of blood and urine sample collection. All subjects whose urinary level of cotinine was $\geq$75 ng/mL were defined as smokers at the time of urine sample collection.

All subjects were asked to donate blood and an overnight urine sample (ending with the first morning void) at the end of the in-person interview. A total of 513 (88% of interviewed) cases and 534 (88% of interviewed) controls provided a blood sample. Blood samples were collected in heparinized (10 mL) and nonheparinized (4 mL) tubes. Heparinized samples were fractioned into plasma, buffy coat, and erythrocytes on the day of the sample collection and were stored at −80°C. When picking up the overnight urine sample, the interviewer asked the subject about use of tobacco products during the past 60 days. Five hundred and thirty-five (92%) of case patients and 543 (90%) of interviewed control subjects donated an overnight urine sample. The urine samples were processed on the day of collection and acidified (400 mg of ascorbic acid per 20 mL of urine) before they were stored at −80°C until analysis.

Laboratory measurements

Arylamine hemoglobin adducts. Erythrocyte fractions, identifiable only by their code numbers, were set

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

on dry ice and sent to the University of Pittsburgh, where they were stored at −30°C until assays for arylamine-Hb adducts were conducted. Hemoglobin adducts of 4-ABP adducts and 2,6-DMA were quantified using the internal standards [methyl-$^2H_6$]-2,6-dimethylaniline and 4-fluoro-4′-aminobiphenyl as described previously (12, 15). Erythrocyte fractions were thawed and 1 mL of each were centrifuged at 1,000 × *g* for 10 minutes to pellet cell debris. The mixture was then dialyzed for 36 hours against 50 volumes of water using a dialysis bath at 4°C and changing dialysis bath water twice to remove noncovalently bound low–molecular weight compounds. The hemoglobin concentration in each sample was determined by Drabkin's method. Each dialyzed sample was then spiked with the internal standards, the sulfinamide bonds between the electrophilic forms of the arylamines and the sulfur of β93-cysteine residue of hemoglobin were hydrolyzed with aqueous NaOH. The freed arylamines were then extracted into hexane and derivatized with pentafluoropropionic anhydride. The resulting amides were concentrated under a stream of dry nitrogen, redissolved in *iso*-octane, and analyzed by GC-NICI-SIM-MS using a Carbowax capillary column.

Urinary total cotinine. Cotinine is a major proximate metabolite of nicotine but has a longer half-life than nicotine. Therefore, cotinine is a better biomarker for daily use of cigarettes than nicotine itself and other nicotine metabolites. In the present study, urinary cotinine was measured by the standard gas chromatographic-mass spectrometric method (GC-MS; ref. 16).

Statistical analysis

In the present study, we included 494 (85% of interviewed) case patients and 499 (83% of interviewed) control subjects with available measurement of arylamine hemoglobin adducts. Bladder cancer cases included in this study were comparable with those excluded from the study in terms of age at diagnosis, body mass index, level of education, and smoking status at reference (all $P > 0.05$). Given the skewed distribution of 4-ABP hemoglobin adducts, formal statistical test was conducted on logarithmically transformed values, and geometric (as opposed to arithmetic) means were presented. The analysis of covariance (ANCOVA) method was applied to identify determinants of 4-ABP in all control subjects. Number of cigarettes smoked per day (0, 1–<10, 10–<20, and 20+) at blood draw and urinary levels of cotinine (0, 1–<100, 100–<500, and 500+ ng/mL) were found to be significantly associated with levels of 4-ABP hemoglobin adducts. Thus, in all statistical analysis that included smokers at the time of blood/urine collection, the latter 2 factors were part of the covariates set in regression models, in addition to gender, age at blood draw, and level of education.

The χ^2 and nonparametric statistics methods were used to examine the difference in distributions of 2,6-DMA hemoglobin adducts across different exposures (i.e., smoking or urinary total cotinine) among controls given the skewed distribution of the data and small percentage (6%) of controls with detectable level of 2,6-DMA adducts. The same methods were used to examine the difference in distributions of 2,6-DMA adducts between cases and controls.

Unconditional logistic regression models were used to examine the associations between adducts and risk of bladder cancer. A high proportion of study subjects (72% controls and 91% cases) in our study had undetectable level of 2,6-DMA adducts. We classified subjects who had detectable level of 2,6-DMA into low (below median of positive values in controls) and high levels of exposure. The median value in controls was determined to be 115.9 pg/g hemoglobin. Similarly, we grouped subjects by quartiles based on the distribution of 4-ABP adducts in controls. The strength of the association between levels of adducts and bladder cancer risk was measured by ORs and their corresponding 95% confidence intervals (CI) and *P* values. We assessed the associations in total subjects and in subgroups defined by cigarette smoking status at blood draw and by smoking history over lifetime.

Statistical analyses were conducted using SAS version 9.2 (SAS Institute) statistical software package. All *P* values are 2-sided. $P < 0.05$ was considered statistically significant.

Results

The mean age (±SD) of case patients at diagnosis of bladder cancer was 63.6 (±9.9), years whereas the mean age of control subjects at interview was 64.0 (±10.0) years ($P = 0.74$). Eighty percent of patients with bladder cancer and 77% of control subjects were men ($P = 0.29$). Case patients had similar distributions as control subjects by levels of education and body mass index (Table 1). More case patients were current smokers and exhibited greater number of pack-years of smoking than controls at the reference date (i.e., 2 years before cancer diagnosis for case patients and 2 years before interview for control subjects; Table 1). However, fewer patients with bladder cancer smoked cigarettes during the past 60 days before blood draw. Case patients and control subjects had comparable levels of total cotinine in urine collected around the same time as the blood draw (Table 1).

2,6-DMA adducts were detected in 136 (26%) of 484 bladder cancer cases and 31 (6%) of 499 control subjects ($P < 0.001$). Among control subjects, there was no statistically significant difference in 2,6-DMA adducts levels between current smokers at blood draw and lifelong nonsmokers or across different levels of smoking (i.e., number of cigarettes per day at blood draw or urinary total cotinine). Among subjects with positive 2,6-DMA adducts, the difference in 2,6-DMA adducts between cases and controls [median: 233.9 in cases vs. 115.9 pg/g hemoglobin in controls; geometric mean: 232.0 (95% CI, 176.3–307.6) in cases vs. 162.2 (95% CI, 89.6–293.6) pg/g hemoglobin] was not statistically significant ($P > 0.13$) (data not shown).

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

Table 1. Distributions of selected characteristics among patients with bladder cancer (cases) and control subjects, The Shanghai Bladder Cancer Case-Control Study, 1995–1998

	No. cases (%)	No. of controls (%)	*P*
Total subjects	484 (100)	499 (100)	
Age at blood draw, y			0.466
<50	64 (13.2)	50 (10.0)	
50–<60	60 (12.4)	65 (13.0)	
60–<70	212 (43.8)	222 (44.5)	
≥70	148 (30.6)	162 (32.5)	
Gender			0.290
Male	385 (79.6)	383 (76.8)	
Female	99 (20.5)	116 (23.3)	
Education			0.518
No formal schooling	39 (8.1)	39 (7.8)	
Primary school	121 (25.0)	123 (24.7)	
Middle school	265 (54.8)	290 (58.1)	
College and above	59 (12.2)	47 (9.4)	
Body mass index, kg/m^2			0.191
<18.5 (underweight)	42 (8.7)	46 (9.2)	
18.5–24.9 (normal)	347 (71.7)	377 (75.6)	
≥25 (overweight and obese)	95 (19.6)	76 (15.2)	
Smoking status at reference date			<0.001
Never smokers	166 (34.3)	223 (44.7)	
Former smokers	76 (15.7)	84 (16.8)	
Current smokers	242 (50.0)	192 (38.5)	
No. of pack-years of smoking			
0 (never smokers)	166 (34.3)	223 (44.7)	0.002
<20	123 (25.4)	115 (23.1)	
20–<40	94 (19.4)	94 (18.8)	
≥40	101 (20.9)	67 (13.4)	
No. of cigarettes smoked/d in the past 60 d before blood draw			<0.001
0 (nonsmokers)	357 (73.8)	309 (61.9)	
<10 cigarettes/d	58 (12.0)	55 (11.0)	
10–<20 cigarettes/d	37 (7.6)	57 (11.4)	
20+ cigarettes/d	32 (6.6)	78 (15.7)	
Lifetime never smokers[a]	158 (32.6)	210 (42.1)	<0.001
Urinary levels of total cotinine, ng/mL			0.109
0 (undetectable)	196 (40.5)	174 (34.9)	
1–<100	139 (28.7)	147 (29.5)	
100–<500	52 (10.7)	68 (13.6)	
≥500	58 (12.0)	79 (15.8)	
Unknown	39 (8.1)	31 (6.2)	

[a]Self-reported nonsmoking at both baseline and blood draw, and urinary cotinine <75 ng/mL.

Compared with subjects who had undetectable adducts, individuals exhibiting low (below median of positive values) and high (above median of positive values) levels had an OR of 4.08 (95% CI, 2.16–7.70) and 7.38 (95% CI, 4.16–13.07), respectively, for bladder cancer after adjustment for potential confounders ($P_{trend} < 0.001$; Table 2). The positive association between 2,6-DMA hemoglobin adducts and bladder cancer risk was present separately in smokers and nonsmokers at blood draw, as well as in lifelong nonsmokers.

Among controls, geometric means of 4-ABP adducts increased with increasing number of cigarettes smoked per day at blood draw as well as increasing levels of urinary cotinine (both $P_{trend} < 0.001$; Table 3). Levels of 4-ABP adducts were comparable between men and women after differences in number of cigarettes per day, and urinary total cotinine between the 2 sexes were taken into account (data not shown). There was no correlation between 4-ABP and 2,6-DMA adducts among control subjects (correlation coefficient = 0.03, $P = 0.764$).

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

Table 2. Level of 2,6-DMA adducts in relation to risk of bladder cancer, The Shanghai Bladder Cancer Case-Control Study 1995–1998

	2,6-DMA adducts (pg/g hemoglobin)			
	Undetectable	Low (<115.9)	High (≥115.9)	P_{trend}
Total subjects				
No. cases/no. controls	348/468	46/15	90/16	
OR (95% CI)[a]	1.00 (ref)	4.03 (2.21–7.36)	7.66 (4.41–13.29)	<0.001
Fully adjusted OR (95%CI)[b]	1.00 (ref)	4.08 (2.16–7.70)	7.38 (4.16–13.07)	<0.001
Current smokers at blood draw				
No. cases/no. controls	96/181	12/5	19/4	
OR (95% CI)[a]	1.00 (ref)	4.61 (1.57–13.58)	9.12 (3.00–27.73)	<0.001
Fully adjusted OR (95%CI)[b]	1.00 (ref)	5.30 (1.16–16.94)	11.00 (3.15–38.42)	<0.001
Nonsmokers at blood draw				
No. cases/no. controls	252/287	34/10	71/12	
OR (95% CI)[a]	1.00 (ref)	3.69 (1.78–7.68)	6.85 (3.61–12.99)	<0.001
Fully adjusted OR (95%CI)[b]	1.00 (ref)	3.85 (1.76–8.41)	6.80 (3.52–13.13)	<0.001
Lifelong nonsmokers[c]				
No. cases/no. controls	110/195	13/6	35/9	
OR (95% CI)[a]	1.00 (ref)	4.00 (1.45–11.02)	6.83 (3.14–14.84)	<0.001
Fully adjusted OR (95%CI)[d]	1.00 (ref)	3.87 (1.39–10.75)	6.90 (3.17–15.02)	<0.001

[a]All ORs were adjusted for age at blood draw, gender, and level of education.

[b]All ORs were further adjusted for smoking status at reference date (never, former, or current smokers), number of cigarettes smoked per day at reference date (continuous), number of year of smoking (continuous), number of cigarettes per day in the past 60 days (0, 1–<10, 10–<20, and ≥20 cigarettes), urinary cotinine levels (0, 1–<100, 100–<500, ≥500 ng/mL, or unknown).

[c]Those who did not smoke cigarettes at reference date or at blood draw, and exhibiting less than 75 ng/mL of urinary total cotinine.

[d]Besides age, gender and level of education, ORs were adjusted for urinary level of total cotinine (0 vs. 1–<75 ng/mL).

Table 4 shows the associations between 4-ABP hemoglobin adducts and risk of bladder cancer. No statistically significant associations were observed in total subjects. However, among lifelong nonsmokers, a dose-dependent, statistically significant association between 4-ABP adducts and bladder cancer was noted ($P_{trend} = 0.009$). Subjects possessing the highest quartile of 4-ABP adducts exhibited an OR of 2.3 (95% CI, 1.23–4.24) relative to those in the lowest 2 quartiles (Table 4).

Both hemoglobin adducts of 2,6-DMA and 4-ABP were independently associated with risk of bladder cancer. A positive association between 2,6-DMA hemoglobin adducts and bladder cancer risk was present in both high and low levels of 4-ABP hemoglobin adducts in all subjects as well as in separate groups stratified smoking status at blood draw or over lifetime. Similarly, the level of 2,6-DMA hemoglobin adducts did not modify the positive association between 4-APB hemoglobin adducts and bladder cancer risk ($P_{interaction} = 0.997$).

Discussion

Earlier, on the basis of data of a study in Los Angeles, California, a high-risk region for bladder cancer, we reported a dose-dependent, statistically significant association between 4-ABP hemoglobin adducts, a validated biomarker for 4-ABP exposure in humans, and risk of bladder cancer unrelated to tobacco use (4). We then examined the relationship between hemoglobin adducts of 9 candidate alkylanilines, a subclass of arylamines of which little is known about their potential carcinogenicity to humans, using the same Los Angeles Bladder Cancer Study data. We observed dose-dependent, statistically significant associations between nonsmoking-related bladder cancer and 3 of the 9 alkylaniline adducts, one of which was 2,6-DMA (12). These latter 3 associations were independent of each other. Now, our 2 sets of novel findings are being confirmed in a low-risk population via a case–control study in Shanghai, China, that was initiated in parallel to our Los Angeles study. The remarkable consistency in results between the Los Angeles and the Shanghai studies strongly implicate that the same class of compounds, namely, the ayrlamines, are responsible for most cases of bladder cancer worldwide.

Cigarette smoking is an important source of 4-ABP. Mainstream cigarette smoke was reported to contain 4-ABP at levels of 2.4 to 4.6 ng per cigarette (unfiltered) and 0.2 to 23 ng per cigarette (filtered; refs. 17, 18). We reported earlier that active smokers of non-Asians in Los Angeles had more than 3-fold 4-ABP hemoglobin adducts than lifelong nonsmokers (75.2 vs. 22.1 pg/g hemoglobin) and the relationship was dose-dependent (4). Consistent with our previous findings, the levels of 4-ABP hemoglobin adducts in the present study population also

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

Table 3. Geometric means of 4-ABP adducts by cigarette smoking and urinary total cotinine levels among control subjects only, The Shanghai Bladder Cancer Case-Control Study 1995–1998

	No. of subjects	Geometric means (95% CI) of 4-ABP adducts (pg/g hemoglobin)
Total	499	19.71 (17.57–22.11)
No. of cigarettes smoked/d in the past 60 d before blood draw		
0 (nonsmokers)	309	16.06 (13.92–18.52)
<10 cigarettes/d	55	16.33 (11.88–22.44)
10–<20 cigarettes/d	57	33.95 (24.85–46.39)
20+ cigarettes/d	78	34.18 (26.18–44.64)
P_{trend}		<0.001
Lifetime nonsmokers[a]	210	15.45 (13.00–18.37)
Urinary levels of total cotinine, ng/mL		
0 (undetectable)	174	15.29 (12.66–18.48)
1–<100	147	17.07 (13.89–20.98)
100–<500	68	28.24 (20.86–38.24)
≥500	79	32.99 (24.91–43.69)
P_{trend}		<0.001
Unknown cotinine level	31	19.97 (12.75–31.28)

[a]Those who did not smoke cigarettes at reference date or at blood draw, and exhibiting less than 75 ng/mL of urinary total cotinine.

increased with increasing number of cigarettes per day or urinary levels of total cotinine (both $P_{trend} < 0.001$). However, the absolute values of 4-ABP hemoglobin adducts in Chinese in Shanghai were much lower in both current smokers (22.43 pg/g hemoglobin) and lifelong nonsmokers (17.73 pg/g hemoglobin) than their non-Asian counterparts in Los Angeles, California. Although we followed the exact protocol for assays and the same standard for 4-ABP in the 2 laboratories that conducted the quantification of 4-ABP hemoglobin adducts, the comparison of absolute values may not be appropriate between the 2 study populations. The smaller difference between smokers and nonsmokers among Shanghai Chinese compared with that among Los Angeles non-Asians might have the following reasons. Cigarette products consumed by Chinese in Shanghai, China, may have lower 4-ABP than those consumed by Non-Hispanic whites and blacks in Los Angeles, California. Although there is lack of specific data on levels of 4-ABP in cigarettes from both markets, the level of tobacco-specific nitrosamines was >20 times higher in U.S. brand cigarettes than in cigarettes domestically manufactured in China (19). Another reason for the different levels of 4-ABP hemoglobin adducts between Shanghai Chinese and Los Angeles non-Asians might be due to the higher prevalence of rapid *N*-acetylation in Chinese than in non-Asians. *N*-Acetylation is a major detoxification pathway of tobacco procarcinogens including 4-ABP (20).

Besides tobacco smoke, there are several nonsmoking sources of 4-ABPs that humans are exposed to. 4-Nitrobiphenyl is a product of incomplete combustion that has been identified as component of kerosene heater emission (21) and diesel engine exhaust (22). Exposure to 4-nitrobiphenyl can result in the production of 4-ABP hemoglobin adducts (23). Therefore, exposure to airborne 4-nitrobiphenyl could result in elevated level of 4-ABP-Hb adducts. Fumes from heated cooking oils contain 4-ABP (24). Chinese women usually prepare food for her family and high-temperature cooking with heated oils is a common food preparation method. Finally, 4-ABP has been detected in commercial hair dyes (25). Women using permanent hair dyes were found at increased risk of bladder cancer in our previous study (26). The elevated level of 4-ABP hemoglobin adducts among lifelong nonsmokers, especially in women, could be the results of exposure to these or as-yet-to-be-identified nonsmoking sources of 4-ABP.

In the present study, patients with bladder cancer showed significantly higher hemoglobin adducts of 2,6-DMA than healthy control subjects in both smokers and nonsmokers. Data on the carcinogenicity of 2,6-DMA on humans are limited. Results from toxicologic studies are consistent with a role for 2,6-DMA in human carcinogenesis (9, 27). 2,6-DMA is shown to induce tumors in nasal cavity and in the liver of rats (8, 28) and has been classified by the IARC as a possible human carcinogen (9). A recent mouse model study suggests that metabolites of 2,6-DMA covalently bind to and produce significant levels of DNA adducts in the bladder, ultimately leading to the malignant transformation of urothelial cells (29). Our previous study showed that the 2,6-DMA-Hb adduct was an independent predictor of bladder cancer risk among non-Hispanic whites in Los Angeles, California (12). The consistent findings between our previous study and this study strongly implicate a role of 2,6-DMA in the development of bladder cancer in humans.

2,6-DMA is a metabolite of some drugs (e.g., lidocaine and xylazine; refs. 30, 31). Human exposure to 2,6-DMA in patients receiving lidocaine for local anesthesia or cardiac arrhythmias has been inferred from the resulting increase in 2,6-DMA hemoglobin adduct levels (30). However, the presence of 2,6-DMA hemoglobin adducts in humans not exposed to lidocaine suggests the existence of other sources of 2,6-DMA exposure in humans (14). 2,6-DMA also is a principal metabolite of the veterinary tranquilizer xylazine (32). Xylazine residues have been found in bovine milk and in different tissues (liver, kidney, etc.) collected from treated cattle (32). In the United States, xylazine is not approved by the U.S. Food and Drug Administration (FDA) for use in food-producing animals but it is unclear whether there is widespread practice of

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

Table 4. Level of 4-ABP adducts in relation to risk of bladder cancer, The Shanghai Bladder Cancer Case Control Study 1995–1998

	4-ABP adducts (pg/g hemoglobin)			
	I/II Q (0–24.5)	III Q (24.5–38.9)	IV Q (38.9+)	P_{trend}
Total subjects				
No. cases/no. controls	256/250	122/125	106/124	
OR (95% CI)[a]	1.00 (ref)	0.96 (0.71–1.30)	0.83 (0.61–1.14)	0.271
Fully adjusted OR (95% CI)[b]	1.00 (ref)	1.04 (0.74–1.46)	1.20 (0.83–1.74)	0.358
Smokers at blood draw				
No. cases/no. controls	34/53	42/54	51/83	
OR (95% CI)[a]	1.00 (ref)	1.20 (0.66–2.17)	0.95 (0.55–1.66)	0.785
Fully adjusted OR (95% CI)[b]	1.00 (ref)	1.39 (0.69–2.77)	1.43 (0.72–2.83)	0.345
Nonsmokers at blood draw				
No. cases/no. controls	222/197	80/71	55/41	
OR (95% CI)[a]	1.00 (ref)	1.09 (0.75–1.60)	1.31 (0.83–2.06)	0.258
Fully adjusted OR (95% CI)[b]	1.00 (ref)	0.99 (0.65–1.49)	1.30 (0.80–2.13)	0.376
Lifelong nonsmokers				
No. cases/no. controls	94/144	33/43	31/23	
OR (95% CI)[a]	1.00 (ref)	1.31 (0.77–2.23)	2.24 (1.21–4.14)	0.011
Fully adjusted OR (95% CI)[c]	1.00 (ref)	1.30 (0.76–2.22)	2.29 (1.23–4.24)	0.009

[a] (ORs were adjusted for age at blood draw, gender, and level of education.

[b]ORs were further adjusted for smoking status at reference date (never, former, and current), number of cigarettes smoked per day at reference date (continuous), number of year of smoking (continuous), number of cigarettes per day in the past 60 days (0, 1–<10, 10–<20, and ≥20 cigarettes), and urinary cotinine levels (0, 1–<100, 100–<500, ≥500 ng/mL, or unknown).

[c]Besides age, gender and level of education, ORs were adjusted for urinary level of total cotinine (0 vs. 1–<75 ng/mL).

non–FDA-approved use of xylazine in China. Finally, 2,6-DMA is used as a chemical intermediate in the manufacture of pesticides, for example, metalaxyl, a systemic fungicide used to control plant diseases. Metalaxyl is widely used to control fungi on a variety of fruit and vegetable crops. Humans are exposed to metalaxyl through ingestion of fruit and vegetables with the pesticide residue (33). The maximum pesticide residue limits in Canada are set at 1.0 ppm for apples and 5 ppm for lettuce and other leafy vegetables (34, 35). There are similar regulatory policy in place in European countries and the United States. However, data regarding the allowable levels of metalaxyl residue on food crops in China are unavailable. Given a strong association between 2,6-DMA hemoglobin adducts and bladder cancer risk, identification of specific sources of 2,6-DMA exposure in the environment should be a high priority.

Strengths of this study included the population-based study design, relatively large sample size, and comprehensively collected data on exposure and genetic determinants of study subjects. The main limitation of the present study was the retrospective nature of the study design. Assessment of 4-ABP and 2,6-DMA hemoglobin adducts was conducted on blood samples taken after cancer diagnosis and/or treatment in cases. It is possible that the postdiagnostic profile in these adducts among the cases does not accurately reflect the group's prediagnostic profile. For example, a large proportion of patients with bladder cancer reduced the amount of cigarettes smoked per day or quit smoking completely following their cancer diagnosis, thus the 4-ABP hemoglobin adducts measured would be lower than expected if blood samples had been collected from patients before their cancer diagnosis. Therefore, the present study might underestimate the effect of the 4-ABP exposure on bladder cancer risk, especially for smokers. Furthermore, the carcinogenic process is believed to take decades to complete, and thus, the relevant exposure periods are far removed from the time of blood draw. It is unknown whether recent exposures in study subjects, as captured by the hemoglobin adducts of 4-ABP and 2,6-DMA, generally reflect exposure levels in decades past. Future studies with a prospective study design can overcome these limitations and establish the temporal relation between exposure to 2,6-DMA and 4-ABP and the risk of developing bladder cancer.

In summary, the findings of the present study show that hemoglobin adducts of 4-ABP and 2,6-DMA are independent risk predictors of bladder cancer for lifelong nonsmokers. These consistent findings in this Chinese population with our previous findings among non-Asians in Los Angeles strongly implicate arylamines as major causal agents of human bladder cancer. Given that cigarette smoking accounts for only approximately 50% of the bladder cancer burden in the United States,

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

identification of nonsmoking-related sources of 4-ABP and 2,6-DMA in the environment should be a high priority.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Tao, M.C. Stern, V.K. Cortessis, M.C. Yu, J.-M. Yuan

Development of methodology: L. Tao, B.W. Day, M.C. Yu, J.-M. Yuan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.W. Day, Y.-B. Xiang, D. Van Den Berg, Y.-T. Gao, M.C. Yu, J.-M. Yuan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Tao, B.W. Day, R. Wang, D.V. Conti, Y.-T. Gao, M.C. Yu, J.-M. Yuan

Writing, review, and/or revision of the manuscript: L. Tao, B.W. Day, Y.-B. Xiang, M.C. Stern, M. Gago-Dominguez, V.K. Cortessis, Y.-T. Gao, M. C. Yu, J.-M. Yuan

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Tao, Y.-B. Xiang, V.K. Cortessis, D. Van Den Berg, M.C. Pike, Y.-T. Gao, M.C. Yu, J.-M. Yuan

Study supervision: B.W. Day, D. Van Den Berg, Y.-T. Gao, J.-M. Yuan

Grant Support

The research study was supported by R01 CA065726 and R01 CA114665, and partially supported by R01 CA080205-10S1 and R01 CA144034, all from the National Cancer Institute, Bethesda, MD.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 27, 2012; revised February 27, 2013; accepted March 19, 2013; published OnlineFirst March 28, 2013.

References

1. IARC. Chemical agents and related occupations. IARC Monogr Eval Carcinog Risks Hum 2012;100:9–562.
2. Bryant MS, Skipper PL, Tannenbaum SR, Maclure M. Hemoglobin adducts of 4-aminobiphenyl in smokers and nonsmokers. Cancer Res 1987;47:602–8.
3. Silverman DT, Devesa SS, Moore LL, Rothman N. Bladder cancer. In: Schottenfeld D, Fraumeni JJ, editors. Cancer epidemiology and prevention. New York: Oxford University Press; 2006. p. 1101–27.
4. Skipper PL, Tannenbaum SR, Ross RK, Yu MC. Nonsmoking-related arylamine exposure and bladder cancer risk. Cancer Epidemiol Biomarkers Prev 2003;12:503–7.
5. Castelao JE, Yuan JM, Skipper PL, Tannenbaum SR, Gago-Dominguez M, Crowder JS, et al. Gender- and smoking-related bladder cancer risk. J Natl Cancer Inst 2001;93:538–45.
6. Jiang X, Yuan JM, Skipper PL, Tannenbaum SR, Yu MC. Environmental tobacco smoke and bladder cancer risk in never smokers of Los Angeles County. Cancer Res 2007;67:7540–5.
7. Tao L, Xiang YB, Wang R, Nelson HH, Gao YT, Chan KK, et al. Environmental tobacco smoke in relation to bladder cancer risk–the Shanghai bladder cancer study [corrected]. Cancer Epidemiol Biomarkers Prev 2010;19:3087–95.
8. U. S. National Toxicology Program. Toxicology and carcinogenesis studies of 2,6-xylidine (2,6-dimethylaniline) (CAS No. 87-62-7) in Charles River CD rats (Feed Studies). Technical Report Series No. 278. Research Triangle Park, NC: National Toxicology Program; 1990.
9. 2,6-Dimethylaniline (2,6-xylidine). IARC Monogr Eval Carcinog Risks Hum 1993;57:323–35.
10. IARC. Monographs on the evaluation of carcinogenic risks to humans. Tobacco smoke and involuntary smoking. Vol. 83. Lyon, France: World Health Organization–International Agency for Research on Cancer; 2004.
11. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer 2003;3:733–44.
12. Gan J, Skipper PL, Gago-Dominguez M, Arakawa K, Ross RK, Yu MC, et al. Alkylaniline-hemoglobin adducts and risk of non-smoking-related bladder cancer. J Natl Cancer Inst 2004;96: 1425–31.
13. Yu MC, Skipper PL, Tannenbaum SR, Chan KK, Ross RK. Arylamine exposures and bladder cancer risk. Mutat Res 2002;506–507: 21–8.
14. Bryant MS, Vineis P, Skipper PL, Tannenbaum SR. Hemoglobin adducts of aromatic amines: associations with smoking status and type of tobacco. Proc Natl Acad Sci U S A 1988;85: 9788–91.
15. Skipper PL. Precision and sensitivity of aminobiphenyl hemoglobin adduct assays in a long-term population study. J Chromatogr B Analyt Technol Biomed Life Sci 2002;778:375–81.
16. Skarping G, Willers S, Dalene M. Determination of cotinine in urine using glass capillary gas chromatography and selective detection, with special reference to the biological monitoring of passive smoking. J Chromatogr 1988;454:293–301.
17. Patrianakos C, Hoffmann D. Chemical studies on tobacco smoke. LXIV. On the analysis of aromatic amines in cigarette smoke. J Anal Toxicol 1979;3:150–4.
18. Hoffmann D, Djordjevic MV, Hoffmann I. The changing cigarette. Prev Med 1997;26:427–34.
19. Wu W, Zhang L, Jain RB, Ashley DL, Watson CH. Determination of carcinogenic tobacco-specific nitrosamines in mainstream smoke from U.S.-brand and non-U.S.-brand cigarettes from 14 countries. Nicotine Tob Res 2005;7:443–51.
20. Yu MC, Skipper PL, Taghizadeh K, Tannenbaum SR, Chan KK, Henderson BE, et al. Acetylator phenotype, aminobiphenyl-hemoglobin adduct levels, and bladder cancer risk in white, black, and Asian men in Los Angeles, California. J Natl Cancer Inst 1994;86: 712–6.
21. Tokiwa H, Nakagawa R, Horikawa K. Mutagenic/carcinogenic agents in indoor pollutants; the dinitropyrenes generated by kerosene heaters and fuel gas and liquefied petroleum gas burners. Mutat Res 1985; 157:39–47.
22. Paputa-Peck MC, Marano RS, Schuetzle D, Riley TL, Hampton CV, Prater TJ, et al. Determination of nitrated polynuclear aromatic hydrocarbons in particulate extracts by capillary column gas chromatography with nitrogen selective detection. Anal Chem 1983;55: 1946–54.
23. Suzuki J, Meguro S, Morita O, Hirayama S, Suzuki S. Comparison of *in vivo* binding of aromatic nitro and amino compounds to rat hemoglobin. Biochem Pharmacol 1989;38:3511–9.
24. Chiang TA, Wu PF, Ko YC. Identification of carcinogens in cooking oil fumes. Environ Res 1999;81:18–22.
25. Turesky RJ, Freeman JP, Holland RD, Nestorick DM, Miller DW, Ratnasinghe DL, et al. Identification of aminobiphenyl derivatives in commercial hair dyes. Chem Res Toxicol 2003;16:1162–73.
26. Gago-Dominguez M, Castelao JE, Yuan JM, Yu MC, Ross RK. Use of permanent hair dyes and bladder-cancer risk. Int J Cancer 2001;91: 575–9.
27. IARC. Monographs on the evaluation of carcinogenic risks to humans Occupational exposures of hairdressers and barbers and personal use of hair colourants. Some hair dyes, cosmetic colourants, industrial dyestuffs and aromatic amines. Vol. 57. Lyon, France: IARC; 1997.
28. Short CR, Joseph M, Hardy ML. Covalent binding of [14C]-2,6-dimethylaniline to DNA of rat liver and ethmoid turbinate. J Toxicol Environ Health 1989;27:85–94.
29. Skipper PL, Trudel LJ, Kensler TW, Groopman JD, Egner PA, Liberman RG, et al. DNA adduct formation by 2,6-dimethyl-, 3,5-

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

dimethyl-, and 3-ethylaniline in vivo in mice. Chem Res Toxicol 2006;19:1086–90.
30. Bryant MS, Simmons HF, Harrell RE, Hinson JA. 2,6-Dimethylaniline–hemoglobin adducts from lidocaine in humans. Carcinogenesis 1994;15:2287–90.
31. Parker RJ, Collins JM, Strong JM. Identification of 2,6-xylidine as a major lidocaine metabolite in human liver slices. Drug Metab Dispos 1996;24:1167–73.
32. Putter J, Sagner G. Chemical studies to detect residues of xylazine hydrochloride. Vet Med Rev 1973;2:145–59.
33. Su Y, Mitchell SH, Mac AntSaoir S. Carbendazim and metalaxyl residues in post-harvest treated apples. Food Addit Contam 2003; 20:720–7.
34. PMR Agency. Established maximum residue limit - Metalaxyl. In: EMRL2008-21 & 33. Ottawa, Ontario, Canada: The Health Canada Pest Management Regulatory Agency; 2008.
35. DEFRA. The pesticides (maximum residue levels in crops, food and feeding stuffs) (England and Wales) (Amendment) (No. 2), Regulations 2002. No. 2723, London, England: HMSO; 2002.

Published OnlineFirst March 28, 2013; DOI: 10.1158/1055-9965.EPI-12-1447

Cancer Epidemiology, Biomarkers & Prevention

AACR American Association for Cancer Research

Elevated 4-Aminobiphenyl and 2,6-Dimethylaniline Hemoglobin Adducts and Increased Risk of Bladder Cancer among Lifelong Nonsmokers——The Shanghai Bladder Cancer Study

Li Tao, Billy W. Day, Bibin Hu, et al.

Cancer Epidemiol Biomarkers Prev 2013;22:937-945. Published OnlineFirst March 28, 2013.