Cancer Research

Urinary Levels of Cigarette Smoke Constituent Metabolites Are Prospectively Associated with Lung Cancer Development in Smokers

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are believed to be among the principal causative agents for lung cancer in smokers, but no epidemiologic studies have evaluated the relationship of PAH uptake and metabolism to lung cancer. In this study, we quantified prediagnostic urinary levels of *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT), a validated biomarker of PAH uptake and metabolism, as well as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL), and cotinine and its glucuronides (total cotinine), validated biomarkers of uptake of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and nicotine, respectively, in relation to lung cancer risk among current smokers in a nested case–control study within a cohort of 18,244 Chinese men in Shanghai, China. Urinary levels of PheT, total NNAL, and total cotinine were significantly higher in cases than controls ($N = 476$ matched pairs). ORs (95% confidence intervals) for lung cancer in the second, third, fourth, and fifth quintiles of PheT were 1.70 (1.00–2.88), 1.07 (0.62–1.84), 1.48 (0.86–2.53), and 2.34 (1.33–4.11), respectively, relative to the lowest quartile ($P_{trend} = 0.023$) after adjustment for self-reported smoking intensity and duration and urinary total NNAL and total cotinine. This study also confirmed that urinary total NNAL and total cotinine are independently related to lung cancer risk. *Cancer Res; 71(21); 6749–57. ©2011 AACR.*

Introduction

Although smoking is the most important causal factor for lung cancer, only a fraction of lifelong smokers develop lung cancer. This interindividual variation in smoking-related lung cancer risk may be determined in part by variability in the uptake and metabolism of tobacco smoke carcinogens. There are more than 70 established carcinogens in cigarette smoke (1). Among these, polycyclic aromatic hydrocarbons (PAH) and tobacco-specific nitrosamines, typified by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are widely considered to be among the most important causative agents for lung cancer.

Multiple PAHs, including 14 rated as having sufficient evidence for carcinogenicity in laboratory animals, are present in cigarette smoke (2, 3). Extensive studies show that cigarette smoke condensate fractions enriched in PAH are carcinogenic to mouse skin and rat lung (4, 5). PAH–DNA adducts have been detected in the lungs of smokers, and the distribution of DNA adducts in the *p53* tumor suppressor gene upon treatment with PAH diol epoxide ultimate carcinogens corresponds to the mutational hot spots in this gene, as found in lung tumors from smokers (6). One representative and widely studied PAH, benzo[*a*]pyrene (BaP), is considered carcinogenic to humans by the International Agency for Research on Cancer (7). A validated PAH biomarker that incorporates both exposure and metabolism by the diol epoxide pathway is *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT), a metabolite of phenanthrene, the simplest PAH with a bay region, a feature closely associated with carcinogenicity. In one relatively small previous study (100 cases and 100 controls), a positive, but statistically nonsignificant association between concentration of serum PheT and lung cancer risk in smokers was reported (8). Epidemiologic data on urinary PAH biomarkers in relation to lung cancer risk in smokers are lacking.

NNK is a strong systemic lung carcinogen in rodents. We previously reported a statistically significant, positive association between levels of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (the sum of which is denoted as total NNAL), metabolites of NNK, in serum or urine and risk of lung cancer in 3 prospective cohorts of smokers, but based on relatively small sample sizes (91 to 155 cases and an equal number of controls per cohort; refs. 8, 9). Because our previous study in 2 cohorts of Chinese men did not simultaneously adjust urinary biomarkers of NNK and PAH, the

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doi: 10.1158/0008-5472.CAN-11-0209

potential confounding effect of PAH on the NNAL–lung cancer association could not be ruled out (9).

Cotinine and its glucuronides (total cotinine) are major metabolites of nicotine, an addictive constituent of cigarette smoke. Although noncarcinogenic, nicotine uptake can influence a smoker's smoking behavior. Smoking behavior and nicotine dependence are found to be associated with lung cancer risk, partially through the influence of variants in genes that encode nicotinic acetylcholine receptors (10–12). Previous studies, including ours, found a statistically significant, positive association between total cotinine in urine or serum and lung cancer risk in smokers (9, 13, 14).

In this study, we simultaneously examined the relationship between urinary levels of PheT, total NNAL, and total cotinine and the risk of developing lung cancer in smokers of the Shanghai Cohort Study. With a larger sample size and 20 years of follow-up, this study overcomes limitations of a previous study that also examined the independent association of these biomarkers in serum and lung cancer risk (8). Findings of this study not only support proposed mechanisms of lung carcinogenesis in smokers, as established by previous research, but also provide crucial information on classes of tobacco smoke constituents that are important in the development of regulatory policy for tobacco products.

Materials and Methods

Subjects

Details of the Shanghai Cohort Study have been previously published (15, 16). In brief, the cohort consisted of 18,244 men (constituting 80% of eligible subjects) enrolled from January 1, 1986, through September 30, 1989, who were between 45 and 64 years of age and resided in 1 of 4 small geographically defined communities in Shanghai, China. In addition to in-person interviews eliciting information on use of tobacco and alcohol, usual diet, and medical history, we collected a 10-mL blood sample and 1 single void urine sample from each participant at baseline. The Shanghai Cohort Study has been approved by the Institutional Review Boards at the University of Minnesota and the Shanghai Cancer Institute.

Identification of incident lung cancer cases and deaths was accomplished through annual in-person reinterviews of all surviving cohort members and routine review of reports from the population-based Shanghai Cancer Registry and from the Shanghai Municipal Vital Statistics Office. As of December 31, 2006, losses to follow-up totaled 839 individuals (4.6%) after 20 years of study.

As of December 31, 2006, 706 cohort participants developed lung cancer. Among them, 574 were smokers, 43 were former smokers, and 89 were never smokers at baseline. Our primary objective in this study required all study subjects to be smokers at the time of enrollment when a spot urine sample was collected. For each case who smoked cigarettes at baseline, we randomly selected 1 control subject from all cohort members who were current smokers at enrollment, free of cancer and alive at the time of cancer diagnosis of the index case. Controls were matched to the index case by age at enrollment ($\pm$2 years), date of biospecimen collection ($\pm$1 month), and neighborhood of residence at recruitment.

Laboratory measurements

Urine samples of all study subjects were retrieved from the biospecimen bank. Specimens from matched control subjects and their index cases were always assayed in the same batch. All urine aliquots were identified only by unique codes and randomly placed in any given batch by laboratory personnel who had no knowledge of the case/control status of the test samples.

Analysis of PheT in urine was conducted essentially as described previously (17), with the following modifications. Analysis was carried out on 0.5 mL of urine, [D_{10}]PheT was used as internal standard, 1,2-dihydroxynaphthalene was used as the UV marker for high-performance liquid chromatography collection, and gas chromatography–mass spectrometry was carried out on a TSQ Quantum instrument (Thermo Scientific). The detection limit of PheT was approximately 0.1 fmol/mL urine. The intraday precision of the assay was 5.6% relative SD (RSD), and the interday precision was 11.1% RSD.

The assay for quantifying total NNAL in urine was identical to the one previously described (9). Briefly, the method involves solid phase extraction of urine with Chem-Elute and Oasis MCX mixed mode cation exchange cartridges followed by quantification by gas chromatography with nitrosamine selective detection (8). The detection limit of total NNAL was 0.04 pmol/mL urine. The intraday precision of the assay was 5.5% RSD, and the interday precision was 7.4% RSD. Quantification of total cotinine (free cotinine plus cotinine *N*-glucuronide) in urine was carried out by gas chromatography–mass spectrometry as previously described (18, 19). The detection limit of total cotinine was 9 pmol/mL urine. The intraday precision of the assay was 1.8% RSD, and the interday precision was 2.8% RSD. Urinary creatinine (Cr) was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis, MN) with a Kodak Ektachem 500 chemistry analyzer.

Of the 574 case–control pairs, 62 cases and 38 controls had insufficient amounts of urine for measurement of all 4 urinary biomarkers—PheT, total NNAL, total cotinine, and creatinine. In addition, 15 cases and 13 controls had missing values for 1 or more of the 4 biomarkers. Sixteen urine samples (5 from cases and 11 from controls) showed urinary total cotinine levels below 35 ng/mL, indicating that they were from nonsmokers at the time of urine collection. All case–control pairs with at least 1 member belonging to the subgroups described above were excluded. Thus, this study included 476 matched case–control pairs.

Statistical analysis

Urinary PheT and total NNAL were expressed in picomoles per milligram creatinine, and total cotinine in nanomoles per milligram creatinine to correct for varying water contents of individual spot urine samples. The distributions of all urinary biomarkers measured were markedly skewed toward high values, which were corrected to a large extent by transformation to logarithmic values. Therefore, formal statistical testing

was carried out on logarithmically transformed values, and geometric (as opposed to arithmetic) means are presented.

The χ^2 test and the t test were used to compare the distributions of selected variables between lung cancer cases and controls. The analysis of covariance (ANCOVA) method (20) was used to examine (i) the difference in the concentrations of urinary biomarkers across varying number of cigarettes smoked per day and (ii) the difference in the levels of urinary PheT, total NNAL, and total cotinine between cases and controls with adjustment for age, year of interview, and year of sample collection.

We used standard statistical methods for analyzing data from matched case–control studies (21). Conditional logistic regression models were used to calculate ORs and their corresponding 95% confidence intervals (CI) and P values. For each urinary biomarker, study subjects were grouped into tertiles or quintiles according to the distributions from control subjects. The linear trend test for the association between levels of biomarkers and lung cancer risk was based on ordinal values of tertile or quintile categories. To assess the independent effects of urinary biomarkers on risk of lung cancer, we simultaneously included urinary PheT, total NNAL, total cotinine, number of cigarettes smoked per day, and number of years of smoking in the logistic regression models. We also used unconditional logistic regression methods to examine the association between urinary total NNAL or total cotinine and risk of lung cancer in former data set and current data set separately and in combination given that the findings of former data set were reported previously with the adjustment for matching factors including age, years of sample collection, and neighborhood of residence at recruitment (9).

Statistical analyses were carried out with SAS software (version 9.2; SAS Institute). All P values reported are 2-sided, and those that were less than 0.05 were considered to be statistically significant.

Results

Of the 476 cases, 315 (66%) were histopathologically confirmed whereas the remaining 161 (34%) were based on clinical diagnosis including radiography or computer-assisted tomography. Among the histopathologically confirmed cases, 153 (49%) were squamous cell cancers, 105 (33%) adenocarcinomas, 22 (7%) small cell cancers, and 35 (11%) other cell types. The mean age ($\pm$ SD) of all case patients at cancer diagnosis was 67.6 $\pm$ 6.5 years. The corresponding figure for matched control subjects at the time of cancer diagnosis of index cases was 67.4 $\pm$ 6.2 years. The average time interval between baseline biospecimen collection and cancer diagnosis was 10.2 $\pm$ 5.3 years, ranging from 1 month to 20.5 years.

Age at recruitment and level of education were comparable for lung cancer cases and controls, whereas body mass index was slightly lower in cases than in controls (Table 1). Compared with controls, men who developed lung cancer had higher numbers of cigarettes smoked per day, number of years of smoking, and number of pack-years at baseline. Cases also exhibited higher levels of smoking between baseline and cancer diagnosis relative to their matched controls (Table 1). The percentage of regular drinkers of alcohol was comparable between cases (57%) and controls (55%). However, among alcoholic drinkers, case patients consumed more drinks per day than controls (Table 1).

Urinary levels of PheT and total NNAL both increased with increasing number of cigarettes smoked per day and levels of urinary total cotinine among control subjects (Table 2). The Spearman correlation coefficients were 0.18 ($P < 0.001$) between urinary PheT and total NNAL, 0.19 ($P < 0.001$) between urinary PheT and total cotinine, and 0.41 ($P < 0.001$) between urinary total NNAL and total cotinine.

Lung cancer cases had statistically significantly higher concentrations of urinary PheT, total NNAL, and total cotinine than control subjects ($P < 0.001$ for all; Table 3). Among the 3 biomarkers measured, levels of total NNAL were the lowest, PheT was intermediate, and total cotinine was the highest in both cases and controls (Table 3).

Table 4 shows that the level of urinary PheT was statistically significantly associated with the increased risk of lung cancer in smokers after adjustment for their self-reported history of smoking. The smoking-adjusted ORs (95% CIs) for lung cancer for the second, third, fourth, and fifth quintiles of urinary PheT were 2.06 (1.25–3.38), 1.23 (0.74–2.04), 1.86 (1.19–2.91), and 3.00 (1.76–5.10), respectively, compared with the lowest quintile ($P_{trend} = 0.001$). Further adjustment for urinary total NNAL and total cotinine diminished but did not materially alter the positive PheT–lung cancer risk association ($P_{trend} = 0.023$).

As previously reported (9), this study showed statistically significantly positive relations for lung cancer risk with urinary total NNAL and total cotinine (Table 5). These positive associations between the former and current data sets were comparable ($P_{difference} = 0.44$ for total NNAL–lung cancer risk association; $P_{difference} = 0.97$ for total cotinine–lung cancer risk association). Additional adjustment for urinary PheT did not materially alter the association between urinary total NNAL or total cotinine and lung cancer risk.

We simultaneously examined number of cigarettes per day, number of years of smoking, urinary PheT, total NNAL, and total cotinine, expressed as continuous variables, with risk of lung cancer. Table 6 shows the ORs for lung cancer associated with an increment of 1 pack of cigarettes smoked per day, 10 years of smoking, or 1 unit in logarithmic value of PheT, total NNAL, and total cotinine (an equivalent of 2.7-fold increment for each urinary biomarker; see details in the footnote of Table 4). All 5 variables were statistically significantly associated with an increased risk of lung cancer even after their simultaneous adjustment (all $P_{trend} < 0.05$). Among the 3 urinary biomarkers, multivariate-adjusted OR for lung cancer was highest for total cotinine (OR = 1.64, 95% CI = 1.34–2.01), intermediate for PheT (OR = 1.41, 95% CI = 1.02–1.93), and lowest for total NNAL (OR = 1.28, 95% CI = 1.02–1.59). There was no evidence for an interaction effect of the 3 urinary biomarkers on lung cancer risk.

We repeated all analyses described above after excluding 34 lung cancer cases with less than 2 years between urine sample collection and lung cancer diagnosis and their matched control subjects. The results based on this subset were almost identical to those based on the entire set of data.

Table 1. Baseline demographic and lifestyle characteristics of current smokers who developed lung cancer (cases) and those who remained cancer free (controls) in the Shanghai Cohort Study, 1986 to 2006

	Controls	Cases	P^a
Number of subjects	476	476	—
Age, y; mean ± SD	57.2 ± 4.9	57.4 ± 5.0	0.536
Body mass index (kg/m^2), mean ± SD	21.9 ± 3.1	21.4 ± 2.8	0.006
Level of education, %			
No formal education	8.8	9.7	0.216
Primary (1–6 y)	33.9	28.6	
Secondary and above	57.3	61.7	
No. of cigarettes/d, mean ± SD	15.6 ± 7.6	20.0 ± 8.4	<0.001
No. of years of smoking, mean ± SD	31.5 ± 10.3	35.4 ± 8.1	<0.001
No. of pack-years of cigarettes at baseline interview, mean ± SD	25.2 ± 15.6	35.8 ± 17.8	<0.001
No. of pack-years of cigarettes from baseline interview to cancer diagnosis, mean ± SD	5.7 ± 4.4	7.3 ± 5.1	<0.001
Alcohol drinking, %			
Nondrinkers	43.3	45.2	0.557
Regular drinkers	56.7	54.8	
No. of drinks/d, mean ± SD	2.6 ± 2.2[b]	3.2 ± 2.7[b]	0.008

[a]2-sided *P* values were based on *t* test for continuous variables or χ^2 test for categorical variables.
[b]Among alcohol drinkers only.

Discussion

This study shows that levels of PheT in urine samples of smokers collected years before cancer diagnosis are independently and statistically significantly associated with the risk of developing lung cancer after adjustment for self-reported smoking intensity and duration and urinary total NNAL and total cotinine. This is the first study to show a significant relationship between lung cancer in smokers and a specific biomarker of PAH uptake and metabolism.

Strong evidence supports a major role for PAH as a primary cause of lung cancer in smokers (22, 23). Carcinogenic PAHs in cigarette smoke include BaP, benz[*a*]anthracene, methylchrysenes, benzofluoranthenes, indeno[1,2,3-*cd*]pyrene, dibenz[*a, h*]anthracene, and several others. Certain PAHs, including some of those in cigarette smoke, are potent locally acting carcinogens that induce lung tumors in rodents (7, 22).

An important metabolic activation pathway of BaP and other carcinogenic PAHs has been described previously (24). In the case of BaP, cytochrome P450s, including P450s 1A1 and 1B1, which are expressed in the lungs of smokers, catalyze the first step to BaP-7,8-epoxide (25–27). This is hydrated with catalysis by microsomal epoxide hydrolase yielding BaP-7,8-diol, which is then oxidized by P450s and other enzymes to

Table 2. Geometric means (95% CIs) of urinary PheT and total NNAL by number of cigarettes smoked per day and quartile levels of urinary total cotinine in current smokers (control subjects only) in the Shanghai Cohort Study, 1986 to 2006

	Controls, *N*	PheT (pmol/mg Cr)[a]	Total NNAL (pmol/mg Cr)[a]
No. of cigarettes per day			
<10	88	25.6 (22.8–28.8)	0.14 (0.12–0.16)
10 to <20	157	27.6 (25.3–30.2)	0.18 (0.16–0.22)
20+	231	29.4 (27.3–31.6)	0.24 (0.22–0.26)
P_{trend}		0.047	<0.001
Urinary total cotinine (nmol/mg Cr)			
1st quartile (<4.1)	119	23.9 (21.6–26.3)	0.14 (0.12–0.16)
2nd quartile (4.1–9.3)	119	27.4 (24.9–30.3)	0.18 (0.16–0.20)
3rd quartile (9.4–16.3)	119	29.0 (26.3–32.0)	0.20 (0.16–0.22)
4th quartile (>16.3)	119	32.6 (29.6–36.0)	0.30 (0.26–0.34)
P_{trend}		<0.001	<0.001

[a]Adjusted for age.

Table 3. Geometric means (95% CIs) of urinary PheT, total NNAL, and total cotinine in current smokers who developed lung cancer (cases) and those who remained cancer free (controls) in the Shanghai Cohort Study, 1986 to 2006

	Controls ($n = 476$)	Cases ($n = 476$)	P^a
PheT (pmol/mg Cr)	28.1 (26.7–29.5)	32.1 (30.5–33.8)	<0.001
Total NNAL (pmol/mg Cr)	0.20 (0.18–0.22)	0.28 (0.26–0.30)	<0.001
Total cotinine (nmol/mg Cr)	7.58 (7.00–8.20)	13.5 (12.5–14.6)	<0.001

[a]The geometric means and *P* values were derived from ANOVA models retaining matched case–control sets, of which controls were matched with index cases on current smoking status, age, neighborhood, and year and month of urine collection.

Table 4. Urinary levels of PheT in relation to risk of lung cancer in current smokers in the Shanghai Cohort Study, 1986 to 2006

	PheT in quintiles					P_{trend}
	1 (lowest)	2	3	4	5 (highest)	
PheT (pmol/mg creatinine)	<17.3	17.3–24.8	24.9–31.5	31.6–42.9	>43.0	
N, controls/cases	96/61	95/107	95/67	95/98	95/143	
OR (95% CI)[a]	1.00	1.90 (1.22–2.96)	1.19 (0.76–1.87)	1.86 (1.19–2.91)	2.93 (1.83–4.67)	<0.001
Smoking-adjusted OR (95% CI)[b]	1.00	2.06 (1.25–3.38)	1.23 (0.74–2.04)	1.86 (1.12–3.09)	3.00 (1.76–5.10)	0.001
Biomarkers-adjusted OR (95% CI)[c]	1.00	1.70 (1.00–2.88)	1.07 (0.62–1.84)	1.48 (0.86–2.53)	2.34 (1.33–4.11)	0.023

[a]ORs were derived from conditional logistic regression models that retained the case–control matched pairs, of which controls were matched to the index cases on current smoking status, age, neighborhood of residence, and year and month of urine collection.
[b]Adjusted for number of cigarettes smoked per day and number of years of smoking at baseline.
[c]Further adjusted for urinary total NNAL and total cotinine.

BaP-7,8-diol-9,10-epoxide (BPDE; refs. 24, 28, 29). The latter reacts easily with DNA, producing a major adduct at the N^2 position of deoxyguanosine (29, 30). BPDE also reacts with H_2O yielding trans, anti-BaP-tetraol, a good indicator of the extent of metabolic activation of BaP by this pathway. We have developed a method to quantify trans, anti-BaP-tetraol in human urine (31, 32). The amounts are quite low and likely impractical for a study such as the one reported here. However, we did observe a significant correlation between levels of trans, anti-BaP-tetraol and PheT in the urine of smokers (33). Measurement of PheT is well suited for large-scale epidemiologic studies. Phenanthrene is the simplest PAH with a bay region, a feature closely associated with carcinogenicity (7), although phenanthrene is generally regarded as noncarcinogenic (34). The metabolism of phenanthrene by the diol epoxide pathway closely parallels that of BaP (35). In this study, we measured urinary PheT by an analytically validated assay (17). Among smokers, urinary PheT increased with increasing number of cigarettes smoked per day. More importantly, urinary PheT was independently associated with the risk of developing lung cancer in smokers even after controlling for self-reported number of cigarettes smoked per day, number of years of smoking, urinary total cotinine (an objective biomarker for nicotine uptake), and urinary total NNAL, a metabolite of the tobacco smoke carcinogen, NNK. Only 1 previous epidemiologic study examined the association between PheT and lung cancer risk in a U.S. population and found a 21% higher level of PheT in serum of smokers who developed lung cancer than smokers who remained free of lung cancer (8). This difference was not statistically significant, possibly as a result of the small study sample size (100 cases and 100 controls).

PAHs are formed during the incomplete combustion of organic material. Human exposure to PAH can come from active or passive tobacco smoking; PheT and other PAH biomarkers are significantly elevated in smokers (7, 36). There are also diffuse environmental sources. Nontobacco sources of PAH exposure include industrial and urban air pollution and diet (7). High occupational exposure can occur during the conversion of coal to coke and coal tar and during the processing and use of products derived from coal tar (7). Many previous studies showed significantly elevated levels of urinary 1-hydroxypyrene, a widely used biomarker of PAH exposure, in the urine of coke-oven workers and others with occupational exposures to PAH (7, 37). Chinese nonsmokers regularly exposed to smoke from burning coal exhibited elevated urinary levels of specific PAH including BaP (38); they were also found to experience significantly elevated risk of lung cancer (39). Another substantial source of exposure to PAH is consumption of grilled or smoked foods or foods grown in PAH-polluted environments (7). Given the ubiquitous nature of PAH in foods, the estimation of their

Table 5. Urinary levels of total NNAL and cotinine in relation to risk of developing lung cancer among current smokers in the Shanghai Cohort Study, 1986 to 2006

	NNAL in tertile[a]				Cotinine in tertile[b]			
	1st (low)	2nd	3rd (high)	P_{trend}	1st (low)	2nd	3rd (high)	P_{trend}
Former data set[c]								
No. of controls/no. of cases	43/20	45/49	42/82		43/13	45/50	42/88	
OR (95% CI)[d]	1.00	2.40 (1.23–4.71)	4.88 (2.48–9.60)	<0.001	1.00	3.91 (1.85–8.27)	7.88 (3.75–16.54)	<0.001
Adjusted OR (95% CI)[e]	1.00	2.00 (0.95–4.23)	2.82 (1.31–6.05)	0.009	1.00	2.29 (1.00–5.21)	2.94 (1.25–6.91)	0.020
PheT-adjusted OR (95% CI)[f,g]	1.00	1.91 (0.90–4.07)	2.72 (1.26–5.86)	0.012	1.00	2.27 (0.99–5.19)	2.99 (1.27–7.05)	0.019
Current data set								
N, controls/cases	115/47	117/115	114/163		115/34	117/95	114/163	
OR (95% CI)[d]	1.00	2.51 (1.63–3.86)	3.77 (2.45–5.82)	<0.001	1.00	2.79 (1.74–4.46)	6.01 (3.83–9.43)	<0.001
Adjusted OR (95% CI)[e]	1.00	1.62 (1.01–2.58)	1.83 (1.12–2.99)	0.023	1.00	2.20 (1.34–3.61)	3.90 (2.36–6.43)	<0.001
PheT-adjusted OR (95% CI)[f,g]	1.00	1.58 (0.99–2.53)	1.78 (1.08–2.91)	0.032	1.00	2.17 (1.32–3.56)	3.80 (2.30–6.29)	<0.001
Both data sets combined								
N, controls/cases	158/67	162/164	156/245		158/47	162/145	156/284	
OR (95% CI)[d]	1.00	2.45 (1.71–3.52)	4.01 (2.79–5.77)	<0.001	1.00	3.04 (2.04–4.52)	6.40 (4.36–9.40)	<0.001
Adjusted OR (95% CI)[e]	1.00	1.67 (1.13–2.47)	1.98 (1.32–2.98)	0.001	1.00	2.22 (1.46–3.37)	3.59 (2.34–5.50)	<0.001
PheT-adjusted OR (95% CI)[f]	1.00	1.62 (1.11–2.41)	1.93 (1.28–2.90)	0.003	1.00	2.18 (1.43–3.32)	3.52 (2.30–5.41)	<0.001

[a]The tertile cutoff values of NNAL were ≤0.105, 0.106–0.214, and ≥0.214 pmol/mg creatinine for the early data set and ≤0.159, 0.160–0.295, and ≥0.295 pmol/mg creatinine for the current data set.

[b]The tertile cutoff values of cotinine were ≤5.84, 5.85–14.19, and ≥14.20 nmol/mg creatinine for the former data set and ≤5.85, 5.86–13.64, and ≥13.65 nmol/mg creatinine for the current data set.

[c]These results were reported previously (9). This analysis excluded 4 cases and 32 controls because of missing data on urinary PheT (see details in Materials and Methods).

[d]ORs were derived from unconditional logistic regression models that also included age, year of sample collection, neighborhood of residence at recruitment, and batch number (for both data sets combined only).

[e]ORs were further adjusted for number of cigarettes per day, number of years of smoking, urinary total cotinine (for total NNAL), and urinary total NNAL (for total cotinine).

[f]In addition to all variables above, ORs were further adjusted for urinary total PheT.

[g]Two-sided *P* value for the difference in the NNAL–lung cancer risk association between the 2 data sets was 0.442 and for the difference in the cotinine–lung cancer risk association between the 2 data sets was 0.970.

Table 6. ORs for lung cancer in relation to self-reported cigarette smoking history and urinary levels of PheT, total NNAL, and total cotinine in current smokers, The Shanghai Cohort Study, 1986 to 2006

Exposure variable	Univariate model[a]		Multivariate model[b]	
	OR (95% CI)	*P*	OR (95% CI)	*P*
No. of cigarettes per day (per 20 cigarettes)	4.03 (2.77–5.86)	<0.001	1.93 (1.27–2.93)	0.002
No. of years of smoking (per 10 y)	2.17 (1.75–2.69)	<0.001	1.94 (1.52–2.47)	<0.001
PheT in $\log_e$ (pmol/mg creatinine)[c]	1.68 (1.30–2.18)	<0.001	1.41 (1.02–1.93)	0.036
Total NNAL in $\log_e$ (pmol/mg creatinine)[c]	1.90 (1.56–2.33)	<0.001	1.28 (1.02–1.59)	0.032
Total cotinine in $\log_e$ (nmol/mg creatinine)[c]	2.13 (1.78–2.55)	<0.001	1.64 (1.34–2.01)	<0.001

[a]Separate conditional logistic models were run for each exposure variable.

[b]The conditional logistic model included simultaneously all exposure variables in the table.

[c]One unit in natural logarithmic value is equivalent to the 2.7-fold increment. ORs in the table, for example, were the equivalents of the comparisons of subjects with 46.7 pmol/mg to those with 17.3 pmol/mg (the cutoff value of the lowest quintile) for PheT, 0.30 pmol/mg to 0.11 pmol for total NNAL, and 9.04 nmol/mg to 3.35 nmol/mg for total cotinine.

dietary consumption is challenging. In this study, we examined but failed to establish any statistical association between intake frequencies of specific foods and urinary levels of PheT (data not shown). The biomarker approach of this study, which quantified PheT in urine, offers an objective surrogate measure of PAH exposure from all sources, including those that await identification. A statistically significant positive association between urinary PheT and lung cancer risk independent of smoking history and urinary total cotinine and total NNAL shows the advantage of this biomarker approach for assessment of PAH exposure plus metabolic activation in smokers.

In the same cohort, we previously reported a statistically significant positive association between urinary total NNAL and lung cancer risk in smokers (9). In this analysis with an additional 325 cases and 346 controls, we confirmed this positive association. The positive association did not differ between the former and current data sets. Further adjustment for urinary PheT did not materially alter the positive association between total NNAL and lung cancer risk, suggesting that total NNAL is an independent risk factor for lung cancer. When all 3 urinary biomarkers were examined simultaneously together with smoking intensity and duration, the association of urinary total NNAL with lung cancer risk was weaker than those for total cotinine and PheT. The weaker association of urinary total NNAL could be due, at least in part, to the relatively low levels of NNK in the smoke of cigarettes used in Shanghai and consequently low levels of urinary total NNAL in this study population (40). The geometric mean of urinary total NNAL among controls of this study was 0.20 pmol/mg creatinine, significantly lower than that of smokers in Singapore (0.66 pmol/mg creatinine) and in the United States (1.36 pmol/mg creatinine; refs. 9, 41).

The results of this study showing a strong positive association between total cotinine level and lung cancer risk confirmed the findings of our previous report as well as those of others (9, 14). Cotinine is a major metabolite of nicotine. Nicotine is the major addictive substance in tobacco smoke, but it is not carcinogenic. However, our findings have shown that urinary levels of total cotinine are informative as an independent and objective measure of *in vivo* exposure to cigarette smoke in general. The independent association between urinary total cotinine and lung cancer risk after adjustment for urinary total NNAL and PheT supports the notion that compounds in tobacco smoke other than NNK and PAH also play a role in the development of lung cancer in smokers. Besides NNK and PAH, multiple established carcinogens have been identified in cigarette smoke, which also has tumor promoting, cocarcinogenic, and inflammatory properties.

One of the strengths of this study is that urinary biomarkers including PheT, total NNAL, and total cotinine were measured in urine samples collected years before cancer diagnosis, thereby ruling out the possibility of a spurious association due to smoking behavior changes in lung cancer patients close to their time of clinical diagnosis. Another strength is the simultaneous measurement of multiple urinary biomarkers representing the uptake and metabolism of PAH, NNK, and nicotine in individual study subjects, thus allowing for the examination of the biomarkers' independent and joint effects on lung cancer risk. The large sample size of the study provided definitive results on the association between these urinary biomarkers and lung cancer risk in male smokers from Shanghai, China.

A potential limitation of this study is that urine samples were collected only once, at baseline, from all subjects. However, our previous study of 70 smokers who smoked 10 or more cigarettes per day over a 1-year period, with sampling every other month, showed relatively constant levels of PheT, total NNAL, and total cotinine, with estimated intraclass correlation coefficients of 76% for total NNAL and 58% for total cotinine in urine, and 56% for PheT in plasma (42). Thus, single measurements from urine somewhat adequately represented individual mean exposure over time. The intraindividual variation in urinary biomarkers measured over a wide time period would diminish the observed association between these urinary biomarkers and lung cancer risk. Therefore, this study with a single assessment at baseline would lead to the underestimation of the true effect of these urinary biomarkers on risk of lung cancer if such intraindividual variation had not existed or multiple measurements of urinary biomarkers at different time points had been used.

In summary, using prospectively collected urine samples from participants of the Shanghai Cohort Study, we showed statistically significant, independent, dose-dependent associations between urinary concentrations of PheT, total NNAL, and total cotinine, biomarkers of uptake and metabolism of PAH, NNK, and nicotine, respectively, and increased risk of lung cancer among smokers with comparable smoking histories. These 3 noninvasive biomarkers with as-yet-to-be identified other biomarkers of tobacco smoke exposure and metabolism potentially can be used to develop an individual-based, predictive model for lung cancer risk in smokers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Ms. Xue-Li Wang of the Shanghai Cancer Institute for supervising the field work of the Shanghai Cohort Study and also the Shanghai Cancer Registry for assistance with identification of cancer outcomes in the Shanghai Cohort Study.

Grant Support

This work was supported by USPHS grants R01 CA129534, R01 CA144034, and CA92025.

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Received January 24, 2011; revised August 29, 2011; accepted August 31, 2011; published OnlineFirst October 25, 2011.

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