Urinary Levels of Tobacco-Specific Nitrosamine Metabolites in Relation to Lung Cancer Development in Two Prospective Cohorts of Cigarette Smokers

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Abstract

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (sum of which is denoted as total NNAL) are metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK and NNAL can induce lung cancer in laboratory animals but human data are limited. The association between prediagnostic levels of urinary total NNAL and risk of lung cancer development was evaluated in two prospective cohorts of Chinese cigarette smokers. We conducted a nested case-control study involving 246 cases of incident lung cancer and 245 cohort controls who were individually matched to the index cases by age, gender, neighborhood of residence at cohort enrollment, and date of urine collection. Urinary levels of total NNAL were significantly associated with risk of lung cancer in a dose-dependent manner. Relative to the lowest tertile, risks associated with the second and third tertiles of total NNAL were 1.43 [95% confidence interval (95% CI), 0.86–2.37] and 2.11 (95% CI, 1.25–3.54), respectively (*P* for trend = 0.005) after adjustment for self-reported smoking history and urinary total cotinine. Smokers in the highest tertiles of urinary total NNAL and total cotinine exhibited a 8.5-fold (95% CI, 3.7–19.5) increased risk for lung cancer relative to smokers with comparable smoking history but possessing the lowest tertiles of urinary total NNAL and total cotinine. Findings of the present study directly link NNK exposure to lung cancer development in humans. [Cancer Res 2009;69(7):2990–5]

Introduction

Lung cancer is one of the most common cancers and causes more than 1 million deaths annually worldwide (1, 2). It is also the leading incident cancer and cause of cancer deaths in the United States, with estimated 215,000 new cases and 161,000 deaths in 2008 (3). Tobacco smoking is the single most important risk factor for lung cancer. In the United States, 90% of lung cancer deaths in men and 75% to 80% of lung cancer deaths in women are attributable to tobacco smoking (4).

Although smoking is the most important causal factor for lung cancer, only a fraction of lifelong smokers develop lung cancer over their lifetime. It is estimated that ~16% of male current smokers and 10% of female current smokers die from lung cancer by 75 years of age (5). This interindividual variation in smoking-related lung cancer risk may be determined in part by individual variability in the uptake and metabolism of tobacco carcinogens. There are >60 established carcinogens in cigarette smoke (6). Among these, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons (PAH) are widely considered to be among the most important causative agents for the development of lung cancer. NNK is a strong systemic lung carcinogen in rodents, but epidemiologic data directly linking NNK or its metabolites to risk of lung cancer in humans are unavailable (7).

When NNK is introduced into virtually any biological system, including human beings, it is converted metabolically to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), also a potent pulmonary carcinogen in rats, by carbonyl reductases and related enzymes (7). NNAL is glucuronidated in humans to produce a mixture of glucuronides, NNAL-Glucs (8). Both NNAL and NNAL-Glucs (sum of which will be designated as total NNAL) can be readily quantified in human urine by gas chromatography with nitrosamine selective detection or by combined liquid chromatography-electrospray ionization-tandem mass spectrometry (9, 10). NNK itself is not detectable in human urine due to its extensive metabolism to NNAL and other products. In this report, we examined the relationship between urinary total NNAL and risk of lung cancer in two prospective cohorts of cigarette smokers who had been followed for up to 20 years.

Materials and Methods

Study population. Subjects were drawn from two prospective cohorts of Asian Chinese: the Shanghai Cohort Study and the Singapore Chinese Health Study (11–13). Briefly, the Shanghai Cohort consisted of 18,244 men (constituting 80% of eligible subjects) enrolled from January 1, 1986 to September 30, 1989, who were between 45 and 64 y of age and resided in one of four 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, a 10-mL blood sample and a single-void urine specimen were collected from each participant at baseline.

The Singapore Chinese Health Study included 63,257 Chinese men and women (representing 85% of eligible subjects) belonging to the two major dialect groups (Cantonese and Hokkien) of Chinese in Singapore. The subjects, who were 45 to 74 y of age and resided in government-built housing estates (86% of all residents resided in such facilities), were enrolled between April 1, 1993 and December 31, 1998. At the time of recruitment, each cohort subject was interviewed in person by a trained interviewer using a structured questionnaire that requested information on

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demographics, lifetime use of tobacco, current consumption of alcoholic beverages, current physical activity, menstrual and reproductive histories (women only), occupational exposure, medical history, and family history of cancer. Information about dietary habits during the past 12 mo was obtained using a validated food frequency questionnaire (14). We requested blood (or buccal cells if blood donation was refused) and single-void urine specimens from a random 3% sample of cohort participants between April 1994 and December 1999. Beginning in January 2000, request for biospecimens was extended to all surviving members of the cohort. During the same time, a follow-up survey conducted by telephone brought up to date subjects' histories on use of tobacco and alcohol, medical and medication histories, and, for women, menopausal status and lifetime use of replacement hormones. By April 2005, all surviving cohort subjects had been contacted for biospecimen donation. Samples were obtained from 32,535 participants, representing 61% of eligible subjects. In summary, for subjects whose urine donation occurred during 1994 to 1999, there is an ~1-y interval between dates when smoking history and urine specimens were collected, whereas the corresponding time interval for the remaining subjects was ~7 mo.

Both cohorts have been actively and passively followed for cancer occurrence and deaths since inception. To date, 769 (4.2%) and 17 (<0.05%) subjects were lost to follow-up in Shanghai and Singapore, respectively (15, 16).

The Shanghai Cohort Study has been approved by the Institutional Review Boards at the University of Minnesota and the Shanghai Cancer Institute. The Singapore Chinese Health Study has been approved by the Institutional Review Boards at the University of Minnesota and the National University of Singapore.

Nested case-control study. We used a nested case-control design to examine the association between urinary total NNAL and lung cancer risk among cigarette smokers in the two Chinese cohorts. The nested case-control set for the Shanghai Cohort was based on a previous study of lung cancer within that cohort (17). Each of 259 incident cases of lung cancer diagnosed by March 1997 was individually matched to three cohort members who were free of cancer and alive at the time of cancer diagnosis of the index case. The matching criteria were age (within 2 y), date of biospecimen collection (within 1 mo), and neighborhood of residence at study enrollment. The present study included only cases who were current smokers at baseline, together with one individually matched control per case who also was a current smoker at baseline. For cases with two or more qualifying controls, one was randomly selected. After exclusion of 99 cases (44 were never or former smokers at baseline, 44 were without any matched controls who were current smokers at baseline, and 11 were depleted of urine samples), 160 lung cancer cases and 160 controls remained.

As of November 2005, there were 99 incident lung cancers among participants of the Singapore Chinese Health Study who donated urine specimens and who were current cigarette smokers at biospecimen collection. For each case, one control subject individually matched to the index case by smoking status at baseline (i.e., current smoker), gender, dialect group (Hokkien, Cantonese), age at enrollment (±3 y), date of baseline interview (±2 y), and date of biospecimen collection (±6 mo) was randomly selected from all cohort members who were alive and free of cancer on the date of cancer diagnosis of the index case.

Laboratory methods. The assay for quantifying total NNAL in urine was performed by a modification of a previously published method (9). 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. The detection limit of NNAL was 0.04 pmol/mL. The intraday precision of the assay was 10.9% relative SD (RSD) and interday precision was 13.7% RSD (9, 18). Quantification of total cotinine (free cotinine plus cotinine *N*-glucuronide) in urine was carried out by gas chromatography-mass spectrometry as previously described (19, 20). Urinary creatinine was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis) with a Kodak Ektachem 500 chemistry analyzer.

Assays for total NNAL failed on six urine samples (two cases and four controls). Cotinine was not measured on four additional subjects (three cases and one control) because their urine samples were depleted after measurement of total NNAL. We further excluded 17 subjects (8 cases and 9 controls) with cotinine levels below 35 ng/mL urine, who most likely were nonsmokers at the time of urine collection. Therefore, the final sample size for the present study consisted of 246 lung cancer cases (155 from the Shanghai Cohort and 91 from the Singapore Cohort) and 245 control subjects (152 from the Shanghai Cohort and 93 from the Singapore Cohort).

Statistical analysis. The χ^2 test and the *t* test were used to compare the distributions of selected variables between lung cancer cases and controls. Urinary total NNAL level was expressed in units of pmol/mg creatinine to correct for varying water contents of individual spot urine samples. The distribution of urinary total NNAL was markedly skewed toward high values, which were corrected to a large extent by transformation to logarithmic values. Therefore, formal statistical testing was performed on logarithmically transformed values, and geometric (as opposed to arithmetic) mean is presented. The analysis of covariance (ANCOVA) method (21) was used to examine (*a*) the difference in urinary total NNAL levels across varying levels of urinary cotinine and (*b*) the difference in urinary total NNAL levels between cases and controls with adjustment for age, year of interview, year of sample collection, gender and dialect group (Singapore subjects only), and study location (Shanghai versus Singapore) when both cohorts were analyzed.

To maximize the number of subjects available for data analysis, we broke the individually matched case-control pairs and the unconditional logistic regression method was used. All matching factors (age, gender, dialect group, year of interview, and year of sample collection) were included in all logistic regression models. To examine the independent role of urinary total NNAL in predicting risk of lung cancer, number of cigarettes per day (continuous), number of years of smoking (continuous), and the concentration of urinary total cotinine (ng/mg creatinine) were included in logistic regression models. We assessed the association between biomarker and lung cancer risk by means of the odds ratio (OR), and its corresponding 95% confidence interval (95% CI) and *P* value. Study subjects were grouped into tertiles according to the distribution of the given biomarker among controls within each cohort. We first analyzed data from each cohort separately. Although the distributions of urinary total NNAL were dissimilar between Shanghai and Singapore, the NNAL-lung cancer risk associations were comparable for the two cohorts ($P = 0.54$). Therefore, results based on both cohorts combined, with adjustment for study location (Shanghai versus Singapore), were presented.

Statistical analyses were carried out using Statistical Analysis System software version 9.1 (SAS Institute). All *P* values reported are two sided, and those that were <0.05 were considered to be statistically significant.

Results

Of the 246 cases, 187 were histopathologically confirmed; there were 76 squamous cell cancers, 64 adenocarcinomas, 17 small cell cancers, 11 non–small cell cancers, and 19 other cell types. The remaining 57 cases were based on clinical diagnosis including radiography or computer-assisted tomography. The mean age (±SD) of all case patients at cancer diagnosis was 65.6 (±6.8) years. The corresponding figure for matched control subjects at the time of cancer diagnosis of index cases was 65.5 (±6.9) years. The average time interval between baseline biospecimen collection and cancer diagnosis was 4.0 (±2.5) years, ranging from 1 month to 10.4 years.

Age at recruitment, level of education, and body mass index were comparable for lung cancer cases and controls within each cohort (Table 1). For both cohorts, individuals who developed lung cancer relative to those who remained cancer-free showed higher numbers of cigarettes per day and number of years of smoking, and these differences within the Shanghai Cohort were statistically significant (Table 1). Urinary total cotinine levels also were higher for cases than controls in both cohorts, but only the difference within the

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 and the Singapore Chinese Health Study

	Shanghai Cohort			Singapore Cohort		
	Cases	Controls	*P**	Cases	Controls	*P**
Total no. subjects	155	152		91	93	
Age at interview (y), mean (SD)	57.9 (4.8)	57.6 (4.7)	0.61	61.1 (6.5)	61.3 (6.4)	0.83
Sex, *n* (%)						
Males	155 (100)	152 (100)	—	80 (87.9)	81 (87.1)	0.87
Females	0 (0)	0 (0)		11 (12.1)	12 (12.9)	
Body mass index (kg/m^2), mean (SD)	21.5 (2.8)	21.8 (3.0)	0.34	21.6 (2.8)	22.0 (2.6)	0.23
Level of education, *n* (%)						
No formal education	18 (11.6)	8 (5.3)	0.12	28 (30.8)	24 (25.8)	0.61
Primary school	61 (39.4)	60 (39.5)		53 (58.2)	55 (59.1)	
Secondary school	76 (49.0)	84 (55.2)		10 (11.0)	14 (15.1)	
No. cigarettes per day, mean (SD)	20.7 (7.7)	15.3 (7.2)	<0.0001	19.5 (12.2)	18.2 (14.4)	0.50
No. years of smoking, mean (SD)	36.7 (8.2)	33.6 (9.1)	0.002	48.2 (10.0)	46.6 (10.7)	0.29
No. pack-years of smoking, mean (SD)	38.4 (16.7)	26.0 (14.4)	<0.0001	47.1 (32.0)	43.2 (33.8)	0.42
Urinary cotinine (ng/mg creatinine), mean (SD)	3,033 (2,244)	1,972 (1,573)	<0.0001	2,873 (1,758)	2,517 (1,825)	0.18
Alcohol drinking, *n* (%)						
Nondrinkers	69 (44.5)	65 (42.8)	0.76	50 (55.0)	64 (68.8)	0.05
Regular drinkers	86 (55.5)	87 (57.2)		41 (45.0)	29 (31.2)	
No. alcoholic drinks per day, mean (SD) †	3.7 (3.2)	2.4 (1.8)	0.002	1.4 (2.2)	1.0 (1.5)	0.32

* *P* values (two-sided) were derived from *t* test (for means) or χ^2 (for frequencies) statistics.
† Among alcohol drinkers only.

Shanghai Cohort was statistically significant (Table 1). The mean concentration of urinary total cotinine was comparable between the two cohorts for either controls ($P = 0.51$) or cases ($P = 0.21$).

Urinary total NNAL level increased with increasing levels of urinary total cotinine in cases and controls of both cohorts (both *P*s for trend < 0.0001). The Spearman's correlation coefficients between urinary total NNAL and cotinine for the Shanghai and Singapore cohort subjects were 0.49 and 0.58, respectively (both *P*s < 0.0001; data not shown).

Lung cancer cases had statistically significantly higher levels of urinary total NNAL than control subjects in both cohorts ($P < 0.001$ and $P = 0.018$; Table 2). Urinary total NNAL concentrations were higher among Singapore cases and controls than their counterparts in Shanghai ($P < 0.001$ in both instances; Table 2).

Table 3 shows that urinary total NNAL and urinary total cotinine independently and significantly predicted risk of lung cancer in smokers from Singapore and Shanghai, who possessed comparable self-reported smoking history. The strength of both sets of associations was dose dependent in the Shanghai and Singapore cohorts separately and combined with adjustment for study location (i.e., Shanghai versus Singapore; Table 3).

Table 4 presents the joint effects of urinary total NNAL and total cotinine on risk of lung cancer in smokers with comparable smoking history. Subjects in the highest versus the lowest tertiles of total NNAL and total cotinine exhibited an 8.5-fold (95% CI, 3.7–19.5) increased risk of lung cancer.

Increased levels of urinary total isothiocyanates and serum β-cryptoxanthin have been found to be associated with decreased risk of lung cancer in the Shanghai Cohort previously (17, 22). Further adjustment for these two factors actually strengthened the positive NNAL-lung cancer association in the Shanghai Cohort Study. The adjusted ORs (95% CIs) for the second and third tertile of total NNAL associated with lung cancer risk were 1.67 (0.83–3.36) and 2.19 (1.08–4.44), respectively, compared with the lowest tertile of NNAL (P for trend = 0.03).

Discussion

This study shows that total NNAL in urine of smokers collected years before cancer diagnosis is significantly associated with their subsequent risk of developing lung cancer. These findings corroborate the large body of laboratory data on NNK carcinogenicity in animals, further strengthening the notion that NNK in tobacco smoke is a major contributor to lung cancer in smokers.

This study also shows that among smokers with comparable smoking history, there is a close to 9-fold variation in subsequent risk of lung cancer between those with high versus low levels of urinary total NNAL and total cotinine. These findings have public health implications. Our results suggest that levels of total NNAL and total cotinine in urine are important predictors of lung cancer risk in cigarette smokers, beyond the predictive indices of smoking intensity (number of cigarettes smoked per day) and duration (number of years of regular smoking). We believe that these two noninvasive biomarkers of tobacco smoke exposure can serve as the starting point of an individual-based, predictive model for lung cancer risk in a smoker. Tobacco smoke contains at least 60 established carcinogens, such as PAHs, 1,3-butadiene, and other volatile carcinogens (6). Inclusion of some of these as-yet-to-be developed biomarkers is likely to be critical in rendering the eventual risk assessment model as a useful tool in predicting the lifetime risk of lung cancer in an individual smoker.

Table 2. Baseline urinary concentrations of total NNAL among current smokers who developed lung cancer (cases) and those who remained cancer-free (controls) in the Shanghai Cohort Study and the Singapore Chinese Health Study

	Cases		Controls		*P**
	n	NNAL (pmol/mg creatinine)	*n*	NNAL (pmol/mg creatinine)	
Shanghai Cohort					
Median (range)	155	0.22 (0.02–4.55)	152	0.15 (0.01–2.23)	<0.0001
Geometric mean (95% CI)	155	0.23 (0.20–0.26)	152	0.15 (0.13–0.17)	<0.0001
Singapore Cohort					
Median (range)	91	0.89 (0.10–3.51)	93	0.59 (0.11–4.56)	0.007
Geometric mean (95% CI)	91	0.89 (0.75–1.05)	93	0.66 (0.56–0.78)	0.018
*P**		<0.0001		<0.0001	

NOTE: The least-squares means were derived from multivariate regression models with age at recruitment, year of interview, year of biospecimen collection, gender and dialect group (for Singapore Cohort only), and study location (Shanghai versus Singapore; for both cohorts combined only) as covariates.

*Two-sided *P* values were derived from Wilcoxon log-rank test (for median) or ANCOVA (for geometric mean).

This study shows that measurements of urinary cotinine and total NNAL at a single time point in a smoker can substantially improve the predictive power of a lung cancer risk assessment model based solely on self-reported smoking history (number of cigarettes smoked per day, number of years of regular smoking). Self-reports of smoking intensity and duration are expected to be imprecise. Information on smoking behavior, such as depth of inhalation and number of puffs per cigarette, is difficult, if not impossible, to assess via a questionnaire-based interview. Furthermore, interview-based assessment of smoking intensity and duration does not capture interindividual variability in metabolism of tobacco carcinogens. Therefore, it is not surprising that appropriately chosen biomarkers would significantly improve the predictive power of a lung cancer risk assessment model based solely on self-reported history of smoking habits.

NNAL is found only in the urine of people who use tobacco products or are exposed to secondhand tobacco smoke because NNK is a tobacco-specific compound, which is not present in the diet or in any other environment. NNK itself is extensively

Table 3. Urinary levels of total NNAL and cotinine in relation to risk of developing lung cancer among current smokers in the Shanghai Cohort Study and the Singapore Chinese Health Study

	Shanghai Cohort			Singapore Cohort			Both cohorts combined		
	Ca/Co*	OR (95% CI)†	OR (95% CI)‡	Ca/Co*	OR (95% CI)†	OR (95% CI)‡	Ca/Co*	OR (95% CI)†	OR (95% CI)‡
NNAL in tertile§									
First (low)	25/51	1.00	1.00‖	18/31	1.00	1.00‖	43/82	1.00	1.00
Second	46/51	1.85 (0.99–3.45)	1.56 (0.79–3.07)	26/31	1.47 (0.66–3.25)	1.39 (0.61–3.17)	72/82	1.68 (1.03–2.74)	1.43 (0.86–2.37)
Third (high)	84/50	3.59 (1.96–6.58)	2.04 (1.02–4.05)	47/31	2.72 (1.27–5.83)	2.64 (1.10–6.34)	131/81	3.14 (1.97–5.01)	2.11 (1.25–3.54)
P for trend		<0.0001	0.04		0.008	0.03		<0.0001	0.005
Cotinine in tertile¶									
First (low)	14/56	1.00	1.00**	17/25	1.00	1.00**	31/81	1.00	1.00
Second	60/50	4.94 (2.46–9.94)	3.17 (1.51–6.64)	24/32	1.08 (0.48–2.46)	0.92 (0.40–2.16)	84/82	2.69 (1.61–4.51)	2.23 (1.31–3.80)
Third (high)	81/46	7.39 (3.68–14.82)	3.76 (1.75–8.06)	50/36	2.11 (0.96–4.60)	1.65 (0.69–3.97)	131/82	4.39 (2.64–7.29)	3.16 (1.82–5.50)
P for trend		<0.0001	0.002		0.04	0.22		<0.0001	<0.0001

* No. cases/no. controls.

† ORs were adjusted for age, year of interview, year of sample collection, gender and dialect group (for Singapore Cohort only), and study location (for both cohorts combined only).

‡ ORs were further adjusted for number of cigarettes per day and number of years of smoking. For NNAL, ORs were further adjusted for urinary cotinine. For cotinine, ORs were further adjusted for urinary total NNAL.

§ The tertile cutoff values of NNAL for the Shanghai Cohort were ≤0.105, 0.106 to 0.209, and ≥0.210 pmol/mg creatinine; for the Singapore Cohort, ≤0.468, 0.469 to 0.819, and ≥0.820 pmol/mg creatinine.

‖ Two-sided *P* for the difference in the NNAL-lung cancer risk association between the two cohorts was 0.54.

¶ The tertile cutoff values of cotinine were ≤1,196, 1,197 to 2,614, and ≥2,615 ng/mg creatinine.

** Two-sided *P* for the difference in the cotinine-lung cancer risk association between the two cohorts was 0.07.

Table 4. Joint effect of urinary total NNAL and cotinine levels on risk of developing lung cancer among current smokers in the Shanghai Cohort Study and the Singapore Chinese Health Study

NNAL in tertile*	Cotinine in tertile*					
	First (Low)		Second		Third (high)	
	Ca/Co †	OR (95% CI) ‡	Ca/Co †	OR (95% CI) ‡	Ca/Co †	OR (95% CI) ‡
First (low)	9/47	1.00	23/25	3.93 (1.54–10.05)	11/10	5.08 (1.63–15.89)
Second	14/24	3.01 (1.11–8.10)	31/32	4.15 (1.70–10.12)	22/26	4.48 (1.78–11.31)
Third (high)	8/10	3.41 (1.03–11.25)	30/25	5.58 (2.25–13.84)	93/46	8.47 (3.69–19.46)

* See the tertile cutoff values of cotinine and NNAL in the footnote of Table 3.

† No. cases/no. controls.

‡ ORs were adjusted for age, year of interview, year of sample collection, gender and dialect group, study location (Shanghai versus Singapore), number of cigarettes smoked per day, and number of years of smoking.

metabolized and cannot be detected in urine. NNK has been classified as carcinogenic to humans (23).

Our results reveal that urinary total NNAL levels were >4-fold higher among smokers in Singapore than in Shanghai. One might wonder if the lower levels of total NNAL in Shanghai versus Singapore smokers were the result of degradation of NNAL in urine during storage. Shanghai samples were collected during 1986 to 1989, whereas the Singapore samples were collected during 1994 to 2005. We believe this is an unlikely scenario. Our experimental data have shown that total NNAL is stable for at least 4 years in urine samples stored at −20°C. Further, mean total NNAL (0.74 pmol/mg creatinine) based on samples collected during the early phase of the Singapore Study (1994–1999) was comparable with that (0.65 pmol/mg creatinine) based on samples collected later (2000–2005; $P = 0.70$). We speculate that the varying levels of urinary total NNAL between smokers in Shanghai versus Singapore may stem from the considerably lower concentrations of NNK in local Chinese brands (western, imported brands were unavailable in Shanghai during 1986–1989 when subjects were recruited into the cohort) relative to the western brands used by most smokers in Singapore (24).

Comparable with results of a prior study conducted in Norway (25), the present study showed an independent, positive association between urinary total cotinine and lung cancer risk. Cotinine is a major metabolite of nicotine. Nicotine is the major additive substance in tobacco smoke but it is not carcinogenic. Therefore, the urinary level of cotinine represents a separate (i.e., aside from self-reports of number of cigarettes/day) and objective measure of *in vivo* exposure to nicotine and cigarette smoke. The independent association between urinary total cotinine and lung cancer risk after adjustment for urinary total NNAL and smoking history supports the notion that compounds in tobacco smoke other than NNK also play a role in the development of lung cancer in smokers. As discussed above, there are at least 60 established carcinogens in cigarette smoke, including PAH and other volatile carcinogens. Strong evidence supports a major role for PAH as causes of lung cancer in smokers (4, 26, 27), although data linking specific PAH biomarkers to lung cancer risk in humans are lacking.

One of the strengths of the present study is that total NNAL was 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. There also is remarkable consistency within our study data. A positive NNAL-lung cancer association of comparable magnitude was observed in both Shanghai and Singapore subjects despite differences in the NNK content of cigarettes smoked.

There is evidence that a single measurement of urinary NNAL closely predicts the average level of NNAL measured over a much longer time period. Our recent study of >50 smokers who smoked 10 or more cigarettes per day over a 1-year period, with sampling every other month, showed relatively constant levels of total NNAL in urine, with an overall average coefficient of variation (CV) of 27.8% (SD of CV = 14.5%; ref. 28). This intraindividual longitudinal variation in urinary total NNAL over a 1-year period was relatively small compared with a 20-fold variation in total NNAL levels among those smokers (28).

Dietary risk or protective factors may confound the NNAL-lung cancer association (29). However, adjustment for urinary total isothiocyanates and serum β-cryptoxanthin, two dietary factors that are inversely related to lung cancer risk in our study population, did not materially alter the association between total NNAL and lung cancer risk (13, 17, 22).

In summary, using prospectively collected urine samples from participants of two Chinese cohorts, we showed a statistically significant, dose-dependent association between urinary total NNAL, a biomarker of NNK exposure, and increased risk of lung cancer among smokers with comparable smoking histories.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. WHO. Tobacco or health: a global status report. Geneva: World Health Organization; 1997. p. 10–48.
2. Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. Lancet 2005;366:1784–93.
3. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
4. Hecht SS. Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst 1999;91:1194–210.
5. Peto R, Darby S, Deo H, Silcocks P, Whitley E, Doll R. Smoking, smoking cessation, and lung cancer in the UK since 1950: combination of national statistics with two case-control studies. BMJ 2000;321:323–9.
6. IARC. Tobacco smoke and involuntary smoking. Monographs on the evaluation of carcinogenic risk to humans, vol. 83. Lyon (France): IARC; 2004. p. 53–119.
7. Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. Chem Res Toxicol 1998;11:559–603.
8. Carmella SG, Le Ka KA, Upadhyaya P, Hecht SS. Analysis of *N*- and *O*-glucuronides of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human urine. Chem Res Toxicol 2002;15:545–50.
9. Carmella SG, Han S, Fristad A, Yang Y, Hecht SS. Analysis of total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human urine. Cancer Epidemiol Biomarkers Prev 2003;12:1257–61.
10. Hecht SS. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. Carcinogenesis 2002;23:907–22.
11. Ross RK, Yuan JM, Yu MC, et al. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. Lancet 1992;339:943–6.
12. Yuan JM, Ross RK, Wang XL, Gao YT, Henderson BE, Yu MC. Morbidity and mortality in relation to cigarette smoking in Shanghai, China. A prospective male cohort study. JAMA 1996;275:1646–50.
13. Yuan JM, Stram DO, Arakawa K, Lee HP, Yu MC. Dietary cryptoxanthin and reduced risk of lung cancer: the Singapore Chinese Health Study. Cancer Epidemiol Biomarkers Prev 2003;12:890–8.
14. Hankin JH, Stram DO, Arakawa K, et al. Singapore Chinese Health Study: development, validation, and calibration of the quantitative food frequency questionnaire. Nutr Cancer 2001;39:187–95.
15. Fan Y, Yuan JM, Wang R, Gao YT, Yu MC. Alcohol, tobacco, and diet in relation to esophageal cancer: the Shanghai cohort study. Nutr Cancer 2008;60:354–63.
16. Koh WP, Yuan JM, Wang R, Seow A, Lee HP, Yu MC. Chronic rhinosinusitis and risk of lung cancer in the Singapore Chinese Health Study. Int J Cancer 2008;123:1398–402.
17. London SJ, Yuan JM, Chung FL, et al. Isothiocyanates, glutathione *S*-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China. Lancet 2000;356:724–9.
18. Carmella SG, Akerkar SA, Richie JP, Jr., Hecht SS. Intraindividual and interindividual differences in metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers' urine. Cancer Epidemiol Biomarkers Prev 1995;4:635–42.
19. Hecht SS, Carmella SG, Murphy SE. Effects of watercress consumption on urinary metabolites of nicotine in smokers. Cancer Epidemiol Biomarkers Prev 1999;8:907–13.
20. Murphy SE, Villalta P, Ho SW, von Weymarn LB. Analysis of [3′,3′-d(2)]-nicotine and [3′,3′-d(2)]-cotinine by capillary liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2007;857:1–8.
21. Winer BJ. Statistical principles in experimental design. 2nd ed. New York: McGraw-Hill; 1971.
22. Yuan JM, Ross RK, Chu XD, Gao YT, Yu MC. Prediagnostic levels of serum β-cryptoxanthin and retinol predict smoking-related lung cancer risk in Shanghai, China. Cancer Epidemiol Biomarkers Prev 2001;10:767–73.
23. IARC. Smokeless tobacco and tobacco-specific nitrosamines. Monographs on the evaluation of carcinogenic risks to humans, vol. 89. Lyon (France): IARC; 2005.
24. 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 Tobacco Res 2005;7:443–51.
25. Boffetta P, Clark S, Shen M, Gislefoss R, Peto R, Andersen A. Serum cotinine level as predictor of lung cancer risk. Cancer Epidemiol Biomarkers Prev 2006;15:1184–8.
26. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer 2003;3:733–44.
27. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 2002;21:7435–51.
28. Joseph AM, Hecht SS, Murphy SE, et al. Relationships between cigarette consumption and biomarkers of tobacco toxin exposure. Cancer Epidemiol Biomarkers Prev 2005;14:2963–8.
29. Koh WP, Yuan JM, Sun CL, Lee HP, Yu MC. Middle-aged and older Chinese men and women in Singapore who smoke have less healthy diets and lifestyles than nonsmokers. J Nutr 2005;135:2473–7.