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Low ratio of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-glucuronides (NNAL-Gluc)/free NNAL increases urothelial carcinoma risk

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ABSTRACT

Cigarette smoking is the most important risk factor for bladder cancer. The compound 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are viewed as biomarkers for cigarette smoking exposure. Therefore, we wanted to explore the effects of these urinary metabolites on urothelial carcinoma (UC) risk. We recruited 127 pairs of UC cases and matched healthy participants for a hospital-based case–control study. Participants completed questionnaires of medical and social information, including smoking history, and provided 50 mL urine samples. Urine samples were analyzed for free NNAL and NNAL-Gluc using the liquid chromatography-tandem mass spectrometry method. Nonparametric analysis and multivariate logistic regression were applied to compare the differences in NNK-related metabolites between UC cases and controls, and to estimate the UC risk associated with certain risk factors. Overall, controls with higher cumulative cigarette smoking exposure had higher total NNAL, free NNAL and NNAL-Gluc. In addition, a decreased NNAL-Gluc/free NNAL ratio corresponded to a significantly increased UC risk. The association between the NNAL-Gluc/free NNAL ratio and UC risk was significant in a dose–response manner. Furthermore, cumulative cigarette smoking exposure was found to interact significantly with low NNAL-Gluc/free NNAL ratio to affect UC risk in this study. This is the first study to conclude that the metabolic products of total NNAL, free NNAL and NNAL-Gluc might be measured as biomarkers of cigarette smoking exposure. Furthermore, the NNAL-Gluc/free NNAL ratio was a better biomarker to evaluate UC risk than total NNAL.

1. Introduction

Urothelial carcinoma (UC) arises exclusively from the urothelium, including the renal pelvis, ureter, bladder and urethra, with bladder cancer being the most common form. Bladder cancer is the most common malignancy of the urinary tract and the ninth most common cancer in Taiwan. Cigarette smoking is a well-established risk factor for bladder cancer and accounts for up to 50% of all incident bladder cancer cases (Strope and Montie, 2008). Our recent study found that cigarette smoking, in terms of smoking duration, daily smoking amount and cumulative smoking in pack–years, caused cigarette smokers to have a significantly higher UC risk than non-smokers in a dose-dependent manner (Pu et al., 2007). This is consistent with the finding in a meta-analysis of 43 published case–control and cohort studies (Zeegers et al., 2000). Cigarette smoke contains more than 60 carcinogenic chemicals and could induce tumorgenesis events or increase proliferation of the bladder epithelium (Johansson and Cohen, 1997). However, the precise mechanism of cigarette smoking causing UC has yet to be determined.

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine-derived tobacco-specific nitrosamine (TSNA), has been linked to tobacco-related cancers in humans (Hecht, 1998). NNK is classified as a "Group 1" carcinogen according to the International Agency for Research on Cancer (Kavvadias et al., 2009). NNK is a procarcinogen that requires metabolic activation by cytochrome P450 enzymes to exert its carcinogenic effects (Richter et al., 2009). NNK is metabolized by carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-

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butanol (NNAL). NNAL itself is a carcinogen and can be detoxified to either O- or N-glucuronide, known as NNAL-glucuronides (NNAL-Gluc); this detoxification is mediated by UDP-glucuronosyltransferase (UGTs) (Tricker et al., 2001; Muscat et al., 2005). NNK has induced pulmonary tumors in various animal studies, independent of the route of administration (Hecht, 1999; Rivenson et al., 1988). Its carcinogenic activity in the lung has been repeatedly shown in many studies, even in low-dose exposure to NNK (Church et al., 2009; Spiegelhalder and Bartsch, 1996). These NNK-related metabolites are ultimately excreted in urine. Total NNAL is only found in tobacco-related products, and its level is a biomarker for NNK uptake (Tulunay et al., 2005). In addition, the ratio of NNAL-Gluc/free NNAL may be another useful biomarker for NNK detoxification in smokers (Carmella et al., 2005). Both NNK and NNAL can be activated through α-hydroxylation to form hydroxyl acid (HA), along with DNA adducts such as 7-methylguanine (N^7MeG) or O^6-methylguanine, and pyridyloxobutyl adducts (Hecht, 1999). Among these DNA adducts, N^7MeG levels are higher in bladder cancer tissue than in adjacent normal bladder epithelium (Saad et al., 2006). Our previous study only recruited 126 UC patients to analyze the levels of urinary NNK metabolic indices and found that the levels of urinary total NNAL (free NNAL plus NNAL-Gluc), free NNAL, HA, and N^7MeG were positively correlated with cigarette smoking (Lee et al., 2008). Here, we conducted a case–control study to assess whether the tobacco-specific nitrosamine NNK metabolites, including total NNAL, free NNAL and NNAL-Gluc, were associated with an increased risk of UC.

2. Materials and methods

2.1. Study participants

We conducted a hospital-based case–control study. The study design has been described previously (Chung et al., 2008). From 2007 to 2009, we recruited 127 UC cases diagnosed with histological confirmation and pathological verification. This was done using routine urological practices, including endoscopic biopsy or surgical resection of urinary tract tumors, followed by histopathological examination by board-certified pathologists. A total of 127 healthy control participants were accrued from a hospital-based pool, including those receiving health examinations at Taipei Medical University Hospital and Taipei Municipal Wan Fang Hospital. Healthy controls were matched to UC cases in terms of age ± 3 years, as well as gender, and had no prior history of cancer. The majority of the study participants (>80%) came from Taipei city and drank tap water. All participants provided informed consent forms before with the interview and biological specimen collection. The Research Ethics Committee of the National Taiwan University Hospital, Taipei, Taiwan, approved the study, and it was consistent with the World Medical Association Declaration of Helsinki.

2.2. Questionnaire interview and biological specimen collection

Well-trained interviewers collected information through a face-to-face interview based on a structured questionnaire. The context of the questionnaire included demographics and socioeconomic characteristics, lifestyle such as cigarette smoking, betel nut chewing, and alcohol, tea and coffee consumption, exposure to potential occupational and environmental carcinogens such as pesticide, and personal and family histories of disease. Spot urine samples of 50 mL were collected at the time of recruitment and immediately transferred to a −20 °C freezer until required for urinary tobacco carcinogen NNK metabolite analysis.

2.3. Analysis of NNK metabolites

Concentrations of urinary NNK metabolites were determined using the liquid chromatography-tandem mass spectrometry method (Lee et al., 2008). The method achieved excellent reproducibility and accuracy. Linearity was observed for NNAL and HA compounds ($R^2 = 0.999$) with detection limits ranging from 0.2 pg to 2.4 pg on column and 0.01 to 0.12 ng/mL in samples injected. Precision and accuracy were determined by analyzing urine from a non-smoker, spiked with 1 ng/mL d_3-NNAL (QC samples). The average intra-day and inter-day variations were 6.2 and 5.5% (n = 5), respectively. Accuracy ranged from 88.0% to 107.1% (n = 5). While QC samples and blank were measured every 10 samples, the calibration standard solution (middle concentration) was measured every 20 samples. Our results satisfied the acceptance criteria, indicating good reproducibility for the determination of each analyzed sample in human urine.

2.4. Statistical analysis

The concentration of urinary metabolites was expressed per milligram creatinine to correct for variation in urine flow. Total NNAL concentration (μg/g creatinine) was the sum of urinary free NNAL and NNAL-Gluc. Nonparametric analysis was applied to compare the differences of total NNAL, free NNAL and NNAL-Gluc between UC cases and controls. Further, we compared the differences of total NNAL, free NNAL and NNAL-Gluc by stratification of UC, age, gender and cumulative cigarette smoking exposure. Multivariate logistic regression was used to assess the relationship between urinary NNK metabolite levels and UC risk. Simultaneously, we developed a multivariate logistic regression to estimate the joint effects of the free NNAL and NNAL-Gluc or the NNAL-Gluc/free NNAL ratio and cumulative cigarette smoking exposure on UC risk, adjusted for other risk factors. For the joint effects analysis, the cutoff points for free NNAL, NNAL-Gluc and cumulative cigarette smoking exposure were the medians of the control group's distributions. After log-transformation of non-normalized variables, Pearson's correlations were adopted to estimate the relationships among urinary total NNAL, free NNAL and NNAL-Gluc. For plots, the values of free NNAL/total NNAL and NNAL-Gluc/total NNAL were algebra-transformed. All data were analyzed using the SAS statistical package. $P<0.05$ was considered significant.

3. Results

The distributions of sociodemographic characteristics and smoking behavior are shown in Table 1. Study participants who had an educational level of college or above had a significantly lower UC risk than those with an elementary school education or below. Ever-smokers (current and former smokers) had a significantly higher UC risk than non-smokers. Cigarette smoking indices in terms of cigarette smoking duration, daily cigarette smoking amount and cumulative cigarette smoking in pack-years, had a significantly higher UC risk than non-smokers in a dose-dependent manner after age and gender adjustment.

Urinary tobacco carcinogen NNK metabolites in UC cases and controls are compared in Table 2. The NNAL-Gluc/free NNAL ratio, was statistically significantly higher in UC cases than in controls. In the control group, males had higher free NNAL and a lower NNAL-Gluc/free NNAL ratio than females. Furthermore, urinary total NNAL, free NNAL and the NNAL-Gluc/free NNAL ratio were significantly associated with cigarette smoking indices in terms of cumulative cigarette smoking exposure (pack-years). There were significantly positive correlations between total NNAL and free NNAL ($r = 0.62$, $p \le 0.01$), and between total NNAL and NNAL-Gluc ($r = 0.92$, $p \le 0.01$). Conversely, there were significantly negative correlations between the ratio of free NNAL/total NNAL and NNAL-Gluc/total NNAL ($r = -0.90$, $p \le 0.01$) (data not shown).

Conducting a stratification analysis on the urinary tobacco carcinogen NNK metabolites profile and ratio indices revealed that NNAL-Gluc or the NNAL-Gluc/free NNAL ratio was significantly

Table 1
Sociodemographic characteristics and smoking status of urothelial carcinoma (UC) cases and age-matched non-cancer controls.

Variables	UC cases N = 127 N (%)	Controls N = 127 N (%)	Age–gender adjusted OR (95% CI)
Age (years)	56.53 ± 1.05	56.64 ± 1.07	1.00 (0.98–1.02)[a]
Gender			
Female	37 (29.13)	37 (29.13)	1.00[b]
Male	90 (70.87)	90 (70.87)	1.00 (0.58–1.72)
Education			
Elementary school or below	38 (29.92)	24 (18.90)	1.00
High school	52 (40.94)	42 (33.07)	0.69 (0.35–1.38)
College or above	37 (29.13)	61 (48.03)	0.33 (0.16–0.67)
Cigarette smoking status			
Non-smoker	66 (52.80)	100 (78.74)	1.00
Ever-smoker	59 (47.20)	27 (21.26)	4.82 (2.55–9.12)
Duration of cigarette smoking (years)			
0	66 (52.80)	100 (78.74)	1.00*
<28	24 (19.20)	14 (11.02)	3.50 (1.57–7.82)
≥28	35 (28.00)	13 (10.24)	6.41 (2.89–14.78)
Amount of cigarette smoking (pack/day)			
0	66 (52.80)	100 (79.37)	1.00*
<0.5	18 (14.40)	14 (11.11)	2.82 (1.23–6.46)
≥0.5	41 (32.80)	12 (9.52)	7.52 (3.44–16.44)
Cumulative cigarette smoking (pack–years)			
0	66 (53.66)	101 (80.16)	1.00*
<19	25 (20.33)	13 (10.32)	3.35 (1.47–7.66)
≥19	32 (26.02)	12 (9.52)	7.05 (3.18–15.66)

One control was unavailable for cigarette smoking; two cases were unavailable for duration of cigarette smoking (years); two cases and 1 control were unavailable for amount of cigarette smoking (pack/day); and 4 cases and 1 control were unavailable for cumulative cigarette smoking (pack-years).
[a] Gender-adjusted odds ratio by logistic regression.
[b] Age-adjusted odds ratio by logistic regression.
* $p<0.05$ for trend test.

associated with UC risk in a dose–response relationship in the multivariate analysis (Table 3). The NNAL-Gluc and the NNAL-Gluc/free NNAL ratios were negatively related to UC risk after adjusting for cumulative cigarette smoking exposure (Table 3) or total NNAL (data not shown).

The joint effects of cumulative cigarette smoking exposure and the urinary NNK metabolite profile on UC risk are shown in Table 4. Significant dose–response relationships were observed in the combination of cumulative cigarette smoking exposure and the ratio of NNAL-Gluc/free NNAL on UC risk.

Table 2
Comparison of urinary tobacco carcinogen NNK metabolites (mean ± standard error) by UC, age, gender, and cumulative cigarette smoking exposure.

Categories	Number	Total NNAL (μg/g creatinine)	Free NNAL (μg/g creatinine)	NNAL-Gluc (μg/g creatinine)	NNAL-Gluc / free NNAL
UC cases	127	1.77 ± 0.49	0.71 ± 0.20	1.18 ± 0.38*	4.43 ± 0.90*
Controls	127	0.64 ± 0.11	0.15 ± 0.02	0.62 ± 0.12*	8.75 ± 1.00*
In cases					
Age					
<57	67	1.45 ± 0.44	0.61 ± 0.20	0.85 ± 0.27	3.46 ± 0.83
≥57	60	2.15 ± 0.94	0.82 ± 0.38	1.56 ± 0.76	5.53 ± 1.66
Gender					
Male	90	1.73 ± 0.64	0.68 ± 0.21	1.07 ± 0.46	4.13 ± 1.10
Female	37	1.86 ± 0.70	0.79 ± 0.49	1.43 ± 0.66	5.16 ± 1.52
Cumulative exposure of cigarette smoking (pack–years)					
0	66	1.28 ± 0.42	0.51 ± 0.28	0.98 ± 0.39	5.35 ± 1.56
<19	20	1.32 ± 0.61	0.73 ± 0.32	0.65 ± 0.35	1.85 ± 0.80
≥19	37	3.06 ± 1.50	1.12 ± 0.46	1.94 ± 1.10	4.29 ± 1.14
In Controls					
Age					
<57	65	0.68 ± 0.20	0.15 ± 0.02	0.66 ± 0.22	7.52 ± 1.14
≥57	62	0.59 ± 0.09	0.15 ± 0.03	0.57 ± 0.10	10.16 ± 1.70
Gender					
Male	90	0.67 ± 0.15	0.17 ± 0.02*	0.63 ± 0.16	7.16 ± 1.03*
Female	37	0.56 ± 0.13	0.09 ± 0.04*	0.58 ± 0.14	12.90 ± 2.27*
Cumulative exposure of cigarette smoking (pack-years)					
0	101	0.47 ± 0.06*	0.13 ± 0.02*	0.46 ± 0.06*	8.89 ± 1.15
<19	13	0.90 ± 0.20*	0.21 ± 0.06*	0.80 ± 0.18*	7.98 ± 3.26
≥19	12	1.74 ± 1.01*	0.19 ± 0.05*	1.70 ± 1.07*	9.21 ± 2.80

*$p<0.05$ through nonparametric analysis, including Kruskal–Wallis or Wilcoxon two-sample analysis.
There are missing total NNAL, free NNAL and NNAL-Gluc data for 5 of the UC cases and free NNAL data for 1 of the controls.

Table 3
Dose–response relationship between urinary tobacco carcinogen NNK metabolites and UC risk.

	UC cases	Controls	Multivariate adjusted
	N = 127	N = 127	OR (95% CI)
Total NNAL (μg/g creatinine)	1.77 ± 0.49	0.64 ± 0.11	1.12 (0.96–1.31)
0	0 (0)	12 (9.45)	
<0.226	58 (47.54)	39 (30.71)	1.00
0.226–0.7	26 (21.31)	38 (29.92)	0.58 (0.30–1.14)
≥0.7	38 (31.15)	38 (29.92)	0.69 (0.36–1.34)
Free NNAL (μg/g creatinine)	0.71 ± 0.20	0.15 ± 0.02	2.37 (1.03–5.46)
0	5 (4.10)	15 (11.9)	1.00
<0.035	46 (37.7)	37 (29.37)	3.30 (0.98–11.15)
0.035–0.136	21 (17.21)	37 (29.37)	1.63 (0.46–5.76)
≥0.136	50 (40.98)	37 (29.37)	3.65 (1.09–12.23)
NNAL-Gluc (μg/g creatinine)	1.18 ± 0.38	0.62 ± 0.12	1.05 (0.93–1.18)
0	13 (10.66)	1 (0.91)	
<0.2	56 (45.90)	37 (33.64)	1.00*
0.2–0.5	24 (19.67)	36 (32.73)	0.34 (0.17–0.70)
≥0.5	29 (23.77)	36 (32.73)	0.29 (0.14–0.61)
NNAL-Gluc/free NNAL ratio	4.43 ± 0.90	8.75 ± 1.00	0.95 (0.91–0.92)
<3.55	92 (73.02)	34 (33.66)	1.00*
3.55–8.63	19 (15.08)	34 (33.66)	0.13 (0.06–0.29)
≥8.63	15 (11.90)	33 (32.67)	0.10 (0.04–0.24)

The cutoff point of urinary tobacco carcinogen NNK metabolites ratio was tertile in the control group's distribution.
Adjusted for age, gender, educational levels and cumulative cigarette smoking exposure (pack-years).
* $p<0.05$ for trend test.

4. Discussion

To our knowledge, this is the first study to evaluate the associations between cigarette smoking exposure and UC risk by measurement of tobacco-specific nitrosamine, including total NNAL, free NNAL and NNAL-Gluc. We previously proved the significant association between smoking behaviors and UC risk (Pu et al., 2007). In the present data, we found that ever-smokers had an approximately 5-fold higher UC risk than non-smokers; further, the levels of 3 major urinary NNK metabolites, total NNAL, free NNAL and NNAL-Gluc, increased incrementally with cumulative cigarette smoking exposure in controls. Smokers with lower the ratio of NNAL-Gluc/free NNAL had higher UC risk than non-smokers with higher NNAL-Gluc/free NNAL ratio. In the present study, NNK-related metabolites might be useful internal biomarkers to reflect cigarette smoking exposure and to avoid the possible measurement error from a questionnaire interview process.

Table 4
Interaction of urinary tobacco carcinogen NNK metabolites and cumulative cigarette smoking exposure on UC risk.

Variables		UC cases/control	Multivariate adjusted
			OR (95% CI)
Free NNAL[a]	NNAL-Gluc		
<0.07	≥0.26	6/16	1.00
<0.07	<0.26	53/48	3.48 (1.11–10.91)
≥0.07	≥0.26	42/39	2.94 (0.92–9.42)#
≥0.07	<0.26	26/24	4.13 (1.23–13.93)
Cumulative exposure of cigarette smoking (pack-years)[b]	NNAL-Gluc/Free NNAL		
0	≥0.51	14/39	1.00*
0	<0.51	52/61	3.16 (1.44–6.97)
>0	≥0.51	9/11	3.67 (1.09–12.37)
>0	<0.51	50/16	14.32 (5.28–38.84)

The cutoff point of free NNAL and NNAL-Gluc was the median of the control group's distribution.
[a] Multivariate adjusted ORs: adjusted for age, gender, educational levels and cumulative cigarette smoking exposure (pack-years).
[b] Multivariate adjusted ORs: adjusted for age, gender, educational levels and total NNAL.
*$p<0.05$ for trend test.

Goniewicz et al. analyzed real-life environments to monitor the half-life of urinary total NNAL for 8 daily smokers and 5 occasional smokers. They concluded that the half-life of total NNAL was 10 to 18 days, and defined urinary total NNAL as an efficient biomarker to detect tobacco smoke exposure, even for assessing second-hand smoke (Goniewicz et al., 2009). One study showed that environmental tobacco smoke (ETS) biomarkers, such as nicotine and cotinine, are higher in non-smokers exposed to tobacco smoke than in unexposed non-smokers (Etter et al., 2000). In addition, non-smoking women exposed to ETS take up and metabolize the tobacco-specific carcinogen NNK, which could increase their risk of lung cancer (Anderson et al., 2001). This connection between ETS and UC risk might be further explored in the future.

Although the carcinogenic activity of NNK and its metabolites in the lung has been proved, there are few studies that explore these chemicals that induce bladder cancer risk. In the present study, the levels of total NNAL of UC patients and controls were 1.77 and 0.64 μg/g creatinine (about 1.4 pmol/mg creatinine), respectively. These results were similar to 1.01–2.02 (min–max: 0.66–2.74) pmol/mg creatinine of Singapore Chinese smokers (Hecht et al., 2004), but lower than those of non-Hispanic Black and White smokers (Muscat et al., 2005). These differences might reflect the variance of actual dose of smoking exposure or the susceptibility of racial diversity. A study by Derby et al. observed ethnic and racial differences in NNK dose and detoxification among 578 smokers in Hawaii. They found that the lower NNK exposure of Japanese-American smokers, compared with white smokers, resulted in a lower lung cancer risk; however, this association was not observed for Native Hawaiian smokers (Derby et al., 2009). Besides ethnic differences and ETS, other factors, such as age, gender, and diet might influence NNK metabolite levels (Derby et al., 2009; Hecht et al., 2004). In the Derby et al. study, age was directly associated with both the NNAL-Gluc level and the NNAL-Gluc/free NNAL ratio (Derby et al., 2009). However, our study did not confirm this relationship. Our results showed that the median value of NNAL-Gluc/free NNAL ratio was significantly higher in women than in men, which was consistent with the findings of the Derby et al. study (Derby et al., 2009). In addition, Hecht et al. found a significantly increased consumption of glucobrassicins with decreasing levels of total NNAL, as well as total NNAL, in urine after adjustment for the number of cigarettes smoked per day (Hecht et al., 2004). However, no association was reported between diet and the level of these NNK metabolites in the Derby et al. results (Carmella et al., 1995; Derby et al., 2009).

The NNAL-Gluc/free NNAL ratio, a marker of NNK detoxification, is detected in the urine of smokers, and it has been studied as a factor relating to carcinogenesis (Carmella et al., 1995; Richie et al., 1997; Derby et al., 2009). NNAL-Gluc is a detoxification product of NNAL and NNK, through the process of NNAL glucuronide formation. The UDP-glucuronosyltransferase (UGT) gene plays an important role in mediating glucuronidation activity (Gallagher et al., 2007). Therefore, individuals with a higher ratio of NNAL-Gluc/free NNAL might be partially protected against the carcinogenic effects of NNAL and NNK, compared to those with a lower ratio (Carmella et al., 1995). We did not find an association between total NNAL and UC risk; however, UC patients had a significantly lower NNAL-Gluc/free NNAL ratio than controls. Furthermore UC risk was significantly increased with a decrease in the NNAL-Gluc/ free NNAL ratio in a dose–response manner. Cumulative cigarette smoking exposure was found to interact significantly with low NNAL-Gluc/free NNAL to affect UC risk in this study. Possibly, the NNAL-Gluc/free NNAL ratio was a better biomarker to evaluate NNK metabolites that induce UC tumorgenesis than total NNAL.

The present study has some limitations that need to be considered when interpreting the results. First, the representativeness of a single spot evaluation of urinary tobacco carcinogen NNK metabolites may be limited. However, the values might be reliable if participants did not experience a change in lifestyle and maintained their homeostatic

metabolism. Second, due to the small sample size, statistical significance should be interpreted with caution and we cannot exclude that the association between urinary tobacco carcinogen NNK metabolites and UC might be the result, and not the cause, of UC.

In summary, this is the first study to conclude that the metabolic products of total NNAL, free NNAL and NNAL-Gluc might be measured as biomarkers of cigarette smoking exposure. In addition, the ratio of NNAL-Gluc/free NNAL may be another useful biomarker for assessing UC risk.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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