Research Article

Cancer Epidemiology, Biomarkers & Prevention

Association between Glucuronidation Genotypes and Urinary NNAL Metabolic Phenotypes in Smokers

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Abstract

Background: The most abundant and potent carcinogenic tobacco-specific nitrosamine in tobacco and tobacco smoke is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *In vivo*, NNK is rapidly metabolized to both the (*R*)- and (*S*)-enantiomers of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which possesses similar carcinogenic properties as NNK. The major detoxification pathway for both NNAL enantiomers is glucuronidation by UDP-glucuronosyltransferase (UGT) enzymes including UGT2B10 and UGT2B17. The goal of the present study was to directly examine the role of UGT genotypes on urinary levels of NNAL glucuronides in smokers.

Methods: NNAL-*N*-Gluc, (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, and free NNAL were simultaneously and directly quantified in the urine of smokers by LC/MS analysis. Genotypes were determined by TaqMan assay using genomic DNA.

Results: The functional knockout polymorphism in the *UGT2B10* gene at codon 67 (Asp>Tyr) was significantly ($P < 0.0001$) associated with a 93% decrease in creatinine-adjusted NNAL-*N*-Gluc. The polymorphic whole-gene deletion of the *UGT2B17* gene was associated with significant ($P = 0.0048$) decreases in the levels of creatinine-adjusted (*R*)-NNAL-*O*-Gluc, with a 32% decrease in the levels of urinary (*R*)-NNAL-*O*-Gluc/(*S*)-NNAL-*O*-Gluc among subjects with the *UGT2B17* (*2/*2) genotype as compared to subjects with the *UGT2B17* (*1/*1) genotype.

Conclusions: These results suggest that functional polymorphisms in *UGT2B10* and *UGT2B17* are associated with a reduced detoxification capacity against NNAL and may therefore affect individual cancer risk upon exposure to tobacco.

Impact: This is the first report to clearly demonstrate strong genotype–phenotype associations between both the *UGT2B10* codon 67 Asp<Tyr genotype and urinary NNAL-*N*-Gluc levels and between the *UGT2B17* copy number variant and urinary (*R*)-NNAL-*O*-Gluc levels in smokers. *Cancer Epidemiol Biomarkers Prev; 25(7); 1175–84. ©2016 AACR.*

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Introduction

Cigarette smoking and smokeless-tobacco exposure are the dominant risk factors for many epithelial cancers including lung, oral, pancreatic, and bladder cancers (1), with 85% to 90% of lung cancer cases directly attributed to tobacco exposure. The relative risk for the development of lung cancer for tobacco smokers is approximately 20 times higher than the risk for nonsmokers (2). Tobacco-specific nitrosamines (TSNA) are produced by nitrosation of tobacco alkaloids, including nicotine, during the tobacco curing and fermentation process and are typically present in substantial amounts in unburned tobacco and in tobacco smoke. The most abundant and potent carcinogenic TSNAs are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN; refs. 3–5; e.g., NNK, 19–10,745 ng/cigarette; NNN, 20–58,000 ng/cigarette; refs. 6, 7). In mainstream cigarette smoke, the total level of NNN plus NNK is 2 to 10 times that of the carcinogenic polycyclic aromatic hydrocarbon, benzo(a)pyrene (4). Tissue-specific carcinogenicity is observed in rodent tissues for NNN and NNK (5, 8–11), with NNK much more potent than NNN in inducing lung adenocarcinomas in rodents (12). NNK is metabolized into intermediates which bind to human lung DNA (13), transform human epithelium *in vitro* (14), and induce nontumorigenic human bronchial epithelial cells to become neoplastically transformed in nude mice after subcutaneous transplantation (15). The lifetime dose of 1.6 mg NNK/kg body weight estimated for the average 2 pack-a-day U.S. smoker (4) is similar to the cumulative dose of 1.8 mg NNK/kg body weight which induced lung tumors in rats (8). Therefore, NNK is strongly implicated as a human lung carcinogen.

Urinary NNK metabolites have been assessed as effective biomarkers of tobacco exposure and cancer risk (7). *In vivo*, NNK is rapidly metabolized to both the (*R*)- and (*S*)-enantiomers of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which possess similar carcinogenic properties as NNK (16). It has been found that (*S*)-NNAL is stereo-selectively retained and has a higher tumorigenicity as compared to (*R*)-NNAL in rat lung (17, 18), and studies in smokeless tobacco users indicate that NNAL may also be stereo-selectively retained in humans (19).

The major detoxification pathway for both NNAL enantiomers is glucuronide conjugation on the nitrogen within the pyridine

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ring (NNAL-*N*-Gluc) or on the hydroxyl group on the NNAL sidechain (NNAL-*O*-Gluc). UGT2B10 was shown to exhibit high *N*-Gluc activity against nicotine and several tobacco-specific nitrosamines including NNAL (20, 21). A functional knockout polymorphism in the *UGT2B10* gene at codon 67 (Asp>Tyr) (rs61750900) was associated with a decrease of approximately 95% in NNAL-*N*-Gluc formation in human liver microsomes (HLM; ref. 22) and large decreases of both nicotine-*N*-Gluc and cotinine-*N*-Gluc formation in the urine of smokers (23). No studies have as yet been performed specifically examining the role of the *UGT2B10* codon 67 polymorphism on urinary NNAL-*N*-Gluc levels *in vivo* in smokers.

Three hepatic UGTs, 2B17, 2B7 and 1A9, have been shown to be active in NNAL-*O*-Gluc formation (24, 25). Studies of NNAL glucuronidation *in vitro* have shown that UGTs 2B7 and 2B17 exhibit preferential glucuronidation activity for (*S*)- and (*R*)-NNAL, respectively, whereas UGT1A9 is relatively non-stereospecific (26). Common functional variants are known to exist for UGTs 2B7 and 2B17. A polymorphic whole-gene deletion of the *UGT2B17* gene has been shown to be associated with significant decreases in the formation of total NNAL-*O*-Gluc and (*R*)-NNAL-*O*-Gluc in HLM (24, 26) and with increased risk for lung adenocarcinoma in women (27). A missense polymorphism at codon 268 (His>Tyr) of the *UGT2B7* gene was associated with a 1.4-fold decrease in total NNAL-*O*-Gluc formation activity in HLM (28).

The goal of this study was to assess the levels of NNAL and its glucuronides in the urine of smokers and to determine whether the levels of NNAL-*N*-Gluc and individual NNAL-*O*-Gluc diastereomers are associated with functional polymorphisms in the *UGT2B10*, *UGT2B17*, and *UGT2B7* genes. Using a sensitive ultra-performance liquid chromatrography-mass spectrometry (LC/MS) method developed for the direct assessment of NNAL, NNAL-*N*-Gluc, and each of the NNAL-*O*-Gluc diastereomers, functional polymorphisms in *UGT2B10* and *UGT2B17* were shown to be associated with the levels of NNAL-*N*-Gluc and (*R*)-NNAL-*O*-Gluc, respectively, in the urine of smokers.

Materials and Methods

Chemicals and materials

NNAL and NNAL-*N*-Gluc standards were purchased from Toronto Research Chemicals. UDP-glucuronic acid (UDPGA) was obtained from Sigma-Aldrich. LC/MS grade solvents including methanol, formic acid, ammonium formate, and ammonium bicarbonate were purchased from Fisher Scientific. MilliQ ultrapure water was used for all analysis. D_4-NNAL was kindly provided by Shantu Amin (Penn State University, State College, PA). D_4-NNAL-*N*-Gluc and D_4-NNAL-*O*-Gluc were biochemically synthesized by incubating D_4-NNAL with UDPGA and bovine liver microsomes, prepared as previously described (21, 26), for 2 hours at 37°C, and then purified by high-performance liquid chromatrography (HPLC).

Subjects and specimens

Spot urine specimens and matching oral buccal cell or blood (10 cc) samples were collected from Caucasian current and former smokers who were a subgroup of healthy control subjects in two case–control studies: 110 self-reported current smokers (smoked at least 100 cigarettes lifetime and had smoked at least one cigarette in the year prior to interview) and 29 former smokers (smoked at least 100 cigarettes lifetime but had not smoked for at least 1 year prior to interview) were from a lung cancer case–control study conducted at the H. Lee Moffitt Cancer Center in Tampa, FL (27), and 87 self-reported current smoker subjects were from a colorectal cancer case–control study conducted at Penn State University College of Medicine (Hershey, PA; refs. 29–32). Oral buccal cell or blood genomic DNA was extracted as previously described (27, 29–32) and urine and DNA specimens were stored at −80°C prior to analysis.

The HLM used for these studies were prepared from normal human liver tissue as described previously (33). Liver microsomes were prepared through differential centrifugation and stored at −80°C.

Genotyping analysis

The *UGT2B10* codon 67 Asp>Tyr polymorphism (rs61750900) was determined by real-time PCR using a custom design TaqMan SNP genotyping assay (w1506724762000; Life Technologies). Because a custom design assay was used, real-time results were confirmed by PCR-RFLP analysis in three subjects of each of the three UGT2B10 genotypes [Asp/Asp (*1/*1), Asp/Tyr (*1/*2), Tyr/Tyr (*2/*2)] using the HinF*I* restriction enzyme, as described previously (20). *UGT2B17* copy number variant (CNV) genotypes were determined by real-time PCR using a CNV genotyping assay (Hs03185327_c; Life Technologies) using RNase P as a control (cat no. 4403326; Life Technologies). The *UGT2B7* codon 268 His>Tyr polymorphism (SNP ID: hCV32449742; rs7439366, rs7438284) was determined by real-time PCR using a predesigned TaqMan SNP genotyping assay (c32449742_20; Life Technologies). All real-time PCR was performed in the Washington State University-Spokane Genomics Core Facility using a Bio-Rad CFX384 real-time PCR machine.

Synthesis and purification of (S)-, (R)-, and D_4-NNAL glucuronide standards

D_4-NNAL-*N*-Gluc and D_4-NNAL-*O*-Gluc were synthesized by using Bovine liver microsomes (5 mg protein/mL reaction) in glucuronidation reactions containing D_4-NNAL and UDPGA and then collected and purified by HPLC as previously described (26). (*S*)-NNAL-*O*-Gluc and (*R*)-NNAL-*O*-Gluc standards were prepared in glucuronidation reactions containing HLM (5 mg protein/mL reaction), racemic NNAL (26) and UDPGA, and were collected and purified by the UPLC method described below with the mass spectrometer disconnected.

LC/MS analysis

A 10-μL aliquot of each urine specimen was spiked with 5 μL of an internal standard mixture that included D_4-NNAL, D_4-NNAL-*N*-Gluc, and D_4-NNAL-*O*-Gluc (0.1 ppm each). After the addition of 10 μL ammonium formate (0.5 mol/L), the mixture was vortexed to mix thoroughly. All precipitate was removed by centrifugation at 16,000 × *g* for 10 minutes at 4°C. The supernatant was transferred into 350 μL conical glass sample vials for LC/MS analysis.

Urine samples prepared as described above were analyzed using an Acquity LC/MS system (Waters Corporation) consisting of an Acquity UPLC pump and an autosampler fitted with an Acquity HSS T3 (100 × 2.1 mm, 1.8 μm) UPLC column and a Xevo G2-S QTof mass spectrometer located within the Washington State University-Spokane Mass Spectrometry Core Facility. LC peak separation was performed at a 25°C column temperature

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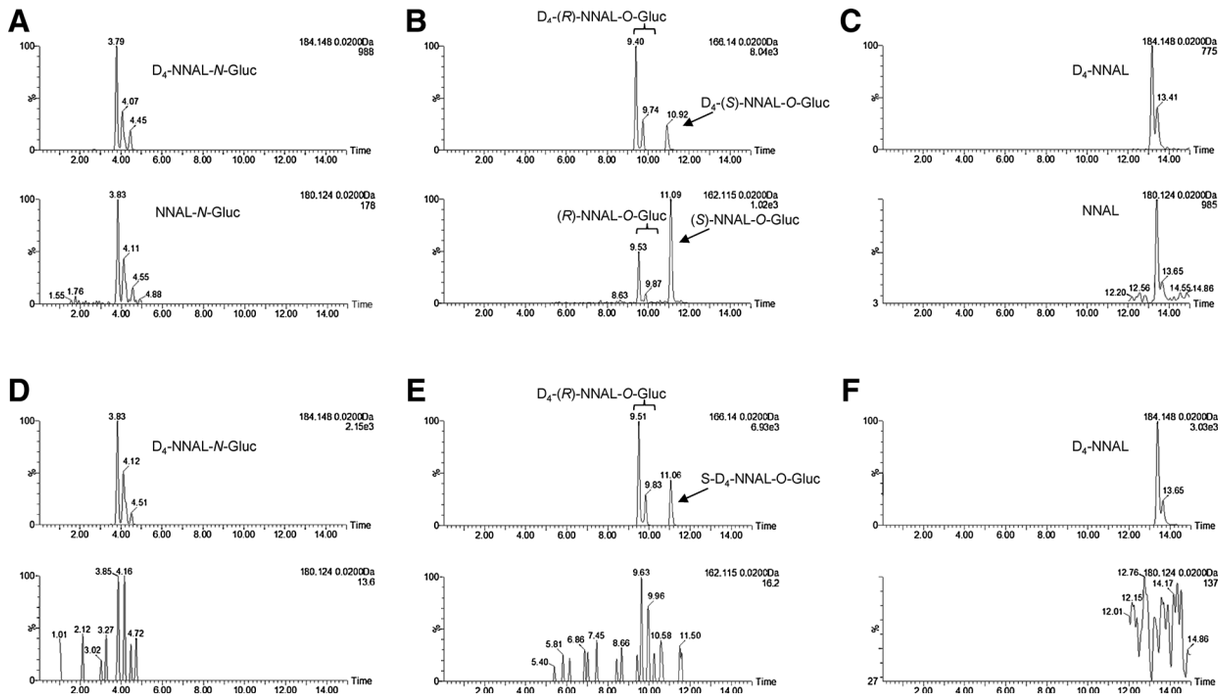

Figure 1.

LC/MS analysis of NNAL and its metabolites in smokers' urine versus former smokers' urine. Urine samples were treated with ammonium formate to normalize the pH and then spiked with the D_4-labeled internal standard (IS) and analyzed as described in the Materials and Methods. Urine from one representative smoker showing clear peaks for NNAL-*N*-Gluc (A), (*R*)-NNAL-*O*-Gluc and (*S*)-NNAL-*O*-Gluc (B), and free NNAL (C) and one former smoker that shows no distinguishable peaks for NNAL-*N*-Gluc (D), (*R*)-NNAL-*O*-Gluc or (*S*)-NNAL-*O*-Gluc (E), or NNAL (F). Internal standards [D_4-NNAL-*N*-Gluc, D_4-(*R*)-NNAL-*O*-Gluc, D_4-(*S*)-NNAL-*O*-Gluc, and D_4-NNAL] are shown in the corresponding top panels.

and the sample injection volume was 5 μL with a flow rate of 0.3 mL/minute using the following conditions: 5 minutes with 99% buffer A (5 mmol/L ammonium formate and 0.01% formic acid in water): 1% buffer B (100% methanol), followed by a linear gradient for 10 minutes to 85% buffer A:15% buffer B, and a subsequent linear gradient for 1 minute to 100% buffer B. Flow continued for 4 minutes in 100% buffer B before reequilibrium in 100% buffer A for 2 minutes. Ammonium bicarbonate at 0.5 mol/L was infused at 10 μL/minute with post column elution flow.

The Waters Xevo G2-S QTof mass spectrometer was operated in positive electrospray ionization MS/MS sensitive mode, with capillary voltage at 0.6 kV. Nitrogen was used for both cone and desolvation gases at 50 and 800 L/hour, respectively. Ultra-pure argon was used as the collision gas with a flow rate of 0.1 L/hour for collision-induced dissociation. The source temperature was 120°C, desolvation gas temperature was 500°C. The dwell time for each ion was 0.1 seconds. The cone voltage was 30 V and the collision energies were 15, 20, and 22 V for NNAL, NNAL-*N*-Gluc, and NNAL-*O*-Gluc, respectively. The MS/MS transition (m/z) traces for quantification for NNAL-*N*-Gluc, NNAL-*O*-Gluc, and NNAL are 386.2 > 180.1 (IS: 390.2 > 184.2), 386.2 > 162.1 (IS: 390.2 > 166.1), and 210.1 > 180.1 (IS: 214.2 > 184.2), respectively.

Urinary creatinine levels were determined after dilution of a small urine specimen 1,000-fold in water, with 10 μL of the diluted sample mixed with 10 μL of 0.1 ppm D3-creatinine for LC/MS analysis using the same LC/MS system described above. LC separation was performed at a 40°C column temperature and the sample injection volume was 2 μL with a flow rate of 0.4 mL/minute using the following conditions: 1 minute with 100% buffer A (5 mmol/L ammonium acetate in water), followed by a linear gradient for 2 minutes to 100% buffer B (100% acetonitrile), and continued for 1 minute in 100% buffer B before re-equilibration in 100% buffer A for 1 minute. Creatinine was detected in positive ionization MS mode with the cone voltage at 20 V. The MS (m/z) trace for quantification of creatinine is 114.06 (IS: 117.08), with a linear range of detection from 2 to 20,000 ppm.

Quantification

Standard curves were constructed by plotting the ratio of analyte [NNAL, (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, NNAL-*N*-Gluc, or creatinine] peak area versus the peak area of the corresponding internal standard, and comparing the observed ratio to a standard curve of eleven analyte concentrations ranging from 0.01 to 10 ppb for NNAL and NNAL metabolites and 2 to 20,000 ppm for creatinine. (*R*)-NNAL-*O*-Gluc and (*S*)-NNAL-*O*-Gluc were quantified independently as described in the Results section. Urinary free NNAL, NNAL-*N*-Gluc, (*S*)-NNAL-*O*-Gluc, (*R*)-NNAL-*O*-Gluc, and creatinine concentrations were calculated using Waters' TargetLynx software.

Statistical analysis

All statistical analysis was performed using Prism version 6.01 (GraphPad Software). Nonparametric Spearman correlation analysis was used to measure the relationship between creatinine-

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adjusted NNAL-*N*-Gluc, (*R*)-NNAL-*O*-Gluc, or (*S*)-NNAL-*O*-Gluc and nicotine-Gluc and 3′-hydroxy (OH)-cotinine-Gluc, which were analyzed in a subset of the same specimens in previous studies (23, 34). Where indicated (when data were not normally distributed), a square root transformation was applied before analysis. The Student *t*-test and the ANOVA linear regression trend test were used to compare the levels of urinary: (i) NNAL-*N*-Gluc in smokers stratified by *UGT2B10* codon 67 Asp>Tyr genotypes, (ii) (*R*)-NNAL-*O*-Gluc stratified by *UGT2B17* CNV genotypes, and (iii) (*S*)-NNAL-*O*-Gluc stratified by *UGT2B7* codon 268 (His>Tyr) genotypes.

Results

Urinary unconjugated NNAL and individual NNAL glucuronides were separated by LC/MS as described in the Materials and Methods. Peaks corresponding to D_4-NNAL-*N*-Gluc, D_4-NNAL-*O*-Gluc, and D_4-NNAL internal standards were observed at 3.79–4.45, 9.53–10.92, and 13.16–13.43 minutes, respectively (Fig. 1A–C). Corresponding NNAL peaks from the urine of smokers matched well with the internal standards, with retention times of 3.83–4.55, 9.53–11.10, and 13.4–13.65 minutes for NNAL-*N*-Gluc, NNAL-*O*-Gluc, and free NNAL, respectively. No NNAL or NNAL glucuronide peaks were observed in the urine specimens from former smokers (Fig. 1D–F).

NNAL exists as two interchangeable *E* and *Z* rotamers of two enantiomers, (*R*) and (*S*). To characterize the glucuronide peaks formed from (*S*)-NNAL versus (*R*)-NNAL using the LC/MS methodology described in this study, (*S*)-NNAL and (*R*)-NNAL were each purified to >99% (26), and were independently incubated with HLM and UDPGA as previously described (21). As observed in Fig. 2, HLM-generated (*S*)-NNAL-*N*-Gluc *E*/*Z* rotamers were observed at retention times of 3.99 and 4.37 minutes (A), whereas the *E*/*Z* rotamers of (*R*)-NNAL-*N*-Gluc were observed at 3.75 and 4.16 minutes (C). HLM-generated (*S*)-NNAL-*O*-Gluc peaks (*E* and *Z*) were observed at a retention time of 11.16 minutes (Fig. 2B). (*R*)-NNAL-*O*-Gluc E/Z rotamer peaks were better separated and observed at 9.53 and 9.86 minutes; a minor (*S*)-NNAL-*O*-Gluc peak was also observed at 11.12 minutes due to a small amount of (*S*)-NNAL in the purified (*R*)-NNAL substrate (Fig. 2D).

NNAL and NNAL glucuronides were analyzed in urine specimens from 197 self-reported current smokers and 29 former smokers. All subjects were of European American decent with an age range of 23 to 80 (mean age = 57 years). Fifty-five percent of the current smokers and 59% of the former smokers were male. Although subjects were recruited from two separate studies, the average age (56 years versus 57 years) and sex distribution (42% female versus 49% female) were similar between studies. The minimum levels of detection of free NNAL, NNAL-*N*-Gluc, (*S*)-NNAL-*O*-Gluc, and (*R*)-NNAL-*O*-Gluc were 0.1, 0.1, 0.025, and 0.05 nmol/L, respectively. The method reproducibility was evaluated by running five randomly-chosen urine specimens in triplicate, performed on three consecutive days. As shown in Table 1, there was good reproducibility for both NNAL-O-Gluc diastereomers and free NNAL. Similar to that observed using other methodologies (35), NNAL-*N*-Gluc determinations were slightly more variable than other metabolites in this study. The assay accuracy was studied by spiking free NNAL, NNAL-*N*-Gluc, (*S*)-NNAL-*O*-Gluc, and (*R*)-NNAL-*O*-Gluc standards into a smoker's urine sample at concentrations of 2.5, 1.25, and 0.625 ppb. The lowest rate of recovery was for NNAL-*N*-Gluc at 83.5% at the lowest concentration tested (0.625 ppb), with the recovery $\geq$90% for all other metabolites at all concentrations examined (Table 2). Linear increases were observed for free NNAL, NNAL-*N*-Gluc, (*S*)-NNAL-*O*-Gluc, and (*R*)-NNAL-*O*-Gluc from 0.04 to 10 ppb (results not shown).

No detectable levels of any of the four NNAL metabolites were found in any of the 29 former smokers' urine specimens tested (results not shown). Of the 197 urine specimens from subjects determined to be "current smokers" based on interview criteria,

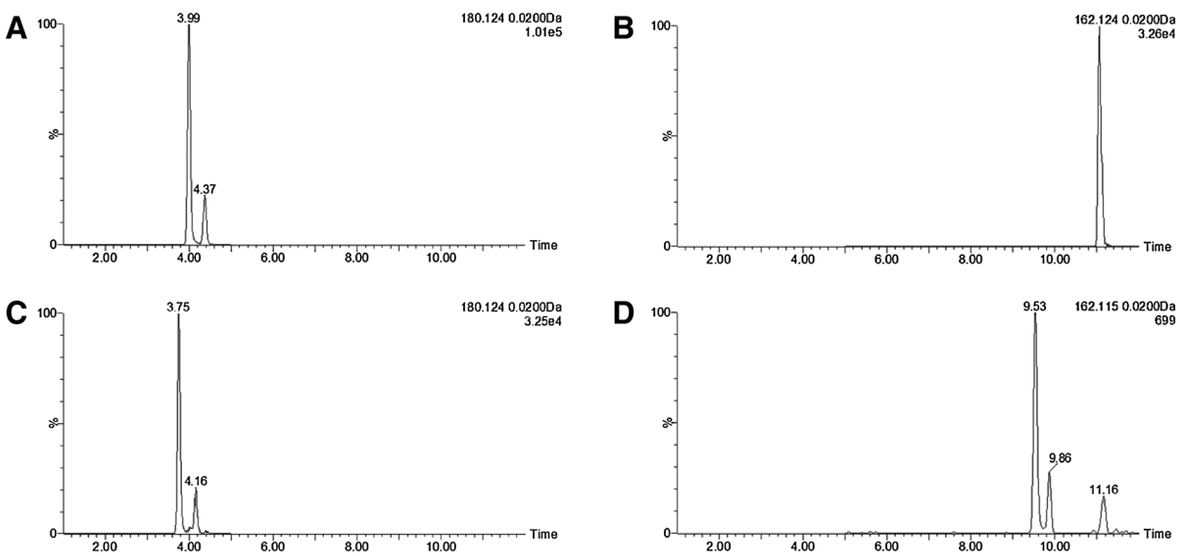

Figure 2.

Human liver microsome incubations with pure NNAL enantiomers. (*S*)- and (*R*)-NNAL were separately incubated with human liver microsomes (1 mg protein/mL reaction) as described in the Materials and Methods. Peaks corresponding to (*S*)-NNAL-*N*-Gluc (A), (*S*)-NNAL-*O*-Gluc (B), (*R*)-NNAL-*N*-Gluc (C), and (*R*)-NNAL-*O*-Gluc (D) are shown.

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Table 1. Reproducibility of method[a]

Specimen	NNAL-*N*-Gluc	(*R*)-NNAL-*O*-Gluc	(*S*)-NNAL-*O*-Gluc	Free NNAL
1	0.61 ± 0.11	0.47 ± 0.08	0.57 ± 0.05	0.64 ± 0.02
2	0.50 ± 0.01	0.45 ± 0.09	0.30 ± 0.01	0.16 ± 0.03
3	Below DL[b]	0.11 ± 0.02	0.15 ± 0.01	0.27 ± 0.04
4	0.43 ± 0.12	0.80 ± 0.07	1.29 ± 0.05	1.57 ± 0.25
5	0.48 ± 0.06	0.90 ± 0.11	0.81 ± 0.03	0.52 ± 0.07

[a]All values are in nmol/L concentrations, shown as mean ± SD of three LC/MS runs per sample analyzed on three consecutive days.
[b]Values were below the detection limit.

several exhibited free NNAL or NNAL metabolite levels that were below the level of detection. To assess the role of UGT genotypes on NNAL metabolite levels in the urine of the 197 subjects determined to be "current smokers" based on interview criteria, only those urine specimens considered to be from "active smokers," previously defined as those subjects with total urinary NNAL (NNAL-*N*-Gluc + (*R*)-NNAL-*O*-Gluc + (*S*)-NNAL-*O*-Gluc + free NNAL) levels of ≥0.23 nmol/L (36), were considered for further analysis. This resulted in the exclusion of 17 urine specimens; of these, no detectable levels of any of the four NNK metabolites examined in this study (free NNAL or any of the three NNAL-Gluc metabolites) were observed in 10 specimens. For the remaining 180 urine specimens, (*S*)-NNAL-*O*-Gluc was detected in 178 subjects, ranging from 0.04 to 5.04 nmol/L; (*R*)-NNAL-*O*-Gluc was detected in 171 subjects with a range of 0.052 to 4.16 nmol/L, NNAL-*N*-Gluc was detected in 161 subjects with a range of 0.10 to 4.76 nmol/L, and free NNAL was detected in 167 subjects with a range of 0.11 to 2.18 nmol/L. These large ranges in urinary NNAL and NNAL-Gluc levels are suggestive of large interindividual differences in NNAL metabolism and excretion.

The average level of urinary creatinine-adjusted free NNAL, (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, and NNAL-*N*-Gluc in the 180 "active smokers" was 0.78, 0.59, 0.72, and 0.62 pmol/mg creatinine, respectively (Table 3). The average percentages of NNAL-*N*-Gluc, (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, and NNAL in total urinary NNAL excreted among the 180 subjects were 23.2%, 21.7%, 26.9%, and 28.2%, respectively. The total creatinine-adjusted NNAL levels were comparable between urine specimens from subjects recruited from the two different studies (2.52 pmol/mg versus 2.84 pmol/mg creatinine), with the average level of urinary creatinine-adjusted free NNAL, (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, and NNAL-*N*-Gluc, in pmol/mg creatinine, at 0.63 versus 0.89, 0.54 versus 0.62, 0.67 versus 0.77, and 0.68 versus 0.57, respectively.

UGT2B10 has been shown to be the primary hepatic enzyme involved in the formation of NNAL-*N*-Gluc (21). UGT2B10 was previously shown to be the major enzyme involved in the glucuronidation of nicotine and the *UGT2B10* codon 67 SNP was correlated with decreased levels of nicotine-*N*-Gluc in the urine of smokers (23). In an analysis of a subset of the smokers' urine specimens ($n = 103$) for whom nicotine-*N*-Gluc and 3′-OH-cotinine-Gluc were measured in previous studies (23, 34) and for whom NNAL metabolites were measured in this study, a significant association was observed between the creatinine-adjusted levels of urinary NNAL-*N*-Gluc and nicotine-*N*-Gluc ($r^2 = 0.46$, $P < 0.0001$; Table 4), which is consistent with UGT2B10 being active in the *N*-Gluc formation of both nicotine and NNAL *in vivo*. Consistent with UGT2B10 not being active in the *in vitro* formation of 3′-OH-cotinine-Gluc and with *UGT2B10* codon 67 genotypes exhibiting no correlation with 3′-OH-cotinine-Gluc levels in smokers' urine in previous studies (34), a much weaker association was observed between the creatinine-adjusted levels of urinary NNAL-*N*-Gluc and 3′-OH-cotinine-Gluc in this study ($r^2 = 0.12$, $P = 0.0005$; Table 3).

An Asp>Tyr SNP at codon 67 of the *UGT2B10* gene has been previously shown to be associated with a loss of enzyme function and decreased hepatic glucuronidation activity (22). Informative genotyping data were obtained for the *UGT2B10* codon 67 polymorphism for 174 of the 180 urinary NNAL metabolite-informative specimens. The allelic prevalence of the Tyr-encoding *UGT2B10*2* allele was 11.5% in the population examined in this study, with four subjects exhibiting the *UGT2B10* (*2/*2) genotype; the allelic distribution of this polymorphism was within Hardy–Weinberg equilibrium ($P = 0.205$). As shown in Fig. 3, there was a strong correlation between *UGT2B10* genotype and creatinine-adjusted NNAL-*N*-Gluc levels in the 174 smokers' urine specimens analyzed in this study (A). Significant decreases of 93% ($P < 0.0001$) and 39% ($P < 0.0001$) were observed for creatinine-adjusted NNAL-*N*-Gluc among subjects with the *UGT2B10* (*2/*2) and (*1/*2) genotypes, respectively, as compared to subjects with the *UGT2B10* (*1/*1) genotype (Fig. 3A). A similarly significant pattern was observed after normalizing by cigarettes/day or after incorporating cigarettes per day in a linear regression model (results not shown). A highly significant ($P = 0.0084$) trend was observed between decreasing creatinine-adjusted NNAL-*N*-Gluc levels and increasing number of the *UGT2B10*2* allele. Similar significant data were observed when comparing *UGT2B10* genotypes versus NNAL-*N*-Gluc/total-NNAL-Gluc (Fig. 3B) or NNAL-*N*-Gluc/(*S*)-NNAL-*O*-Gluc (Fig. 3C).

UGTs 2B7 and 2B17 have been shown to be among the primary hepatic enzymes involved in the formation of NNAL-*O*-Gluc (24, 34), with recent studies demonstrating that UGT2B17 is more active against (*R*)-NNAL than (*S*)-NNAL whereas UGT2B7 is more active against (*S*)-NNAL (26). These enzymes were also shown to be active in the formation of 3′-OH-cotinine-Gluc but not

Table 2. Recovery of urinary NNAL metabolites

	NNAL-*N*-Gluc ($n = 3$)		*R*-NNAL-*O*-Gluc ($n = 3$)		*S*-NNAL-*O*-Gluc ($n = 3$)		NNAL ($n = 3$)	
ppb added	Detected	Recovery	Detected	Recovery	Detected	Recovery	Detected	Recovery
2.5	2.67	101%	3.12	113%	2.95	107%	2.64	96.3%
1.25	1.39	100%	1.71	114%	1.38	89.8%	1.47	98.8%
0.625	0.66	83.5%	1.02	118%	0.93	107%	0.81	92.5%
0	0.14	—	0.29	—	0.26	—	0.24	—

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Table 3. Creatinine-adjusted NNK metabolite levels (pmol/mg creatinine) in the urine of smokers ($n = 180$)[a]

	NNAL-*N*-Gluc	(*R*)-NNAL-*O*-Gluc	(*S*)-NNAL-*O*-Gluc	Total NNAL-Gluc	Free NNAL	Total NNAL
Mean	0.62 ± 0.49	0.59 ± 0.44	0.72 ± 0.50	1.93 ± 1.23	0.78 ± 0.60	2.71 ± 1.61
Range	0.06–3.23[b]	0.10–2.78[c]	0.06–2.96[d]	0.14–6.31	0.11–3.29[e]	0.26–9.45

[a]All concentrations listed are in pmol/mg creatinine (mean ± SD) for 180 metabolite-informative subjects.
[b]$n = 161$; 19 subjects were below the detection limit for NNAL-*N*-Gluc.
[c]$n = 171$; 9 subjects were below the detection limit for (*R*)-NNAL-*O*-Gluc.
[d]$n = 178$; 2 subjects were below the detection limit for (*S*)-NNAL-*O*-Gluc.
[e]$n = 167$; 13 subjects were below the detection limit for free NNAL.

nicotine-Gluc (20, 23, 34). Consistent with the *in vitro* activity observed for UGT2B17 against (*R*)-NNAL, a stronger correlation was observed between the levels of creatinine-adjusted urinary (*R*)-NNAL-*O*-Gluc and 3′-OH-cotinine-Gluc ($r^2 = 0.24$, $P < 0.0001$; Table 4) than that observed between creatinine-adjusted urinary (*R*)-NNAL-*O*-Gluc and nicotine-*N*-Gluc ($r^2 = 0.075$, $P = 0.005$; Table 4). Consistent with the *in vitro* activity observed for UGT2B7 against (*S*)-NNAL, a stronger correlation was also observed between the levels of creatinine-adjusted urinary (*S*)-NNAL-*O*-Gluc and 3′-OH-cotinine-Gluc ($r^2 = 0.35$, $P < 0.0001$; Table 4) than that observed between creatinine-adjusted urinary (*S*)-NNAL-*O*-Gluc and nicotine-Gluc ($r^2 = 0.12$, $P = 0.0004$; Table 4).

The *UGT2B17* whole-gene deletion polymorphism has been previously shown to be associated with decreased 3′-OH-cotinine-Gluc formation in HLM (34), with urinary 3′-OH-cotinine-Gluc levels in smokers (23) and with decreased NNAL-*O*-Gluc formation in HLM (24). Informative genotyping data were obtained for the *UGT2B17* CNV for all 180 urinary NNAL metabolite-informative specimens. A gene deletion *UGT2B17**2 allelic prevalence of 37.5% was observed in this population with 25 subjects exhibiting the *UGT2B17* (*2/*2) genotype. The allele distribution was within Hardy–Weinberg equilibrium ($P = 0.82$). As shown in Fig. 4, there were significant correlations between *UGT2B17* genotype and (*R*)-NNAL-*O*-Gluc/total NNAL-Gluc (panel B; $P = 0.018$) and (*R*)-NNAL-*O*-Gluc/(S)-NNAL-*O*-Gluc (panel C; $P = 0.0048$) in the 180 smokers' urine specimens, with a 32% decrease in the levels of urinary (*R*)-NNAL-*O*-Gluc/(*S*)-NNAL-*O*-Gluc among subjects with the *UGT2B17* (*2/*2) genotype as compared to subjects with the *UGT2B17* (*1/*1) genotype (Fig. 4C). Significant trends toward decreasing levels of urinary (*R*)-NNAL-*O*-Gluc/total NNAL-Gluc ($P_{trend} = 0.034$) and (*R*)-NNAL-*O*-Gluc/(*S*)-NNAL-O-Gluc ($P_{trend} = 0.027$) was observed in subjects with increasing numbers of the *UGT2B17**2 allele (panels B and C, respectively).

Of the 168 subjects for whom informative *UGT2B7* codon 268 (His>Tyr) data were obtained, 25% ($n = 42$) exhibited the homozygous wild-type *UGT2B7* His/His (*1/*1) genotype, 46% ($n = 78$) exhibited the heterozygous wild-type *UGT2B7* (*1/*2) genotype, and 29% ($n = 48$) exhibited the homozygous *UGT2B7* (*2/*2) genotype, a distribution that was consistent with Hardy–Weinberg equilibrium ($P = 0.36$). No significant association was found between the *UGT2B7* codon 268 genotype and urinary (*R*)- or (*S*)-NNAL-*O*-Gluc levels, whether examined as urinary concentrations or as a ratio with total NNAL-Gluc (data not shown).

Table 4. Spearman Correlation coefficients between creatinine-adjusted NNAL glucuronides (pmol/mg creatinine) and creatinine-adjusted nicotine-Gluc or 3′-OH-cotinine-Gluc (pmol/mg creatinine) in 103 smokers' urine[a]

	NNAL-*N*-Gluc	(*R*)-NNAL-*O*-Gluc	(*S*)-NNAL-*O*-Gluc
Nicotine-Gluc	0.46 (4.1E−15)	0.075 (5.0E−3)	0.12 (3.9E−4)
3′-OH-cotinine-Gluc	0.12 (4.5E−4)	0.24 (1.2E−7)	0.35 (3.6E−11)

[a]Values represent r^2, with *P*-values in parenthesis.

Discussion

This study is the first to directly examine the role of functional variants in *UGT2B7*, *UGT2B10*, and *UGT2B17* in NNK metabolism in smokers. Previous studies have shown that although both UGT2B10 and UGT1A4 exhibit NNAL-*N*-Gluc formation activity, UGT2B10 exhibited a 6-fold lower K_m for NNAL than UGT1A4 (21) and the functional polymorphism at codon 67 of the *UGT2B10* gene resulted in an 11-fold decrease in NNAL-*N*-Gluc formation in HLM (22). Results from this study are highly consistent with these *in vitro* data, with smokers homozygous for the *UGT2B10**2 variant exhibiting a 23-fold decrease in the levels of urinary NNAL-*N*-Gluc in comparison to smokers exhibiting the wild-type *UGT2B10* (*1/*1) genotype. Therefore, it is estimated that UGT2B10 is responsible for more than 90% of NNAL-*N*-Gluc formation *in vivo*, indicating that UGT1A4 may be responsible for less than 10% of the elimination of NNAL by NNAL-*N*-Gluc formation in smokers with the wild-type *UGT2B10* (*1/*1) genotype.

The data from this study are also consistent with UGT2B10 playing an important role in the hepatic *N*-glucuronidation and excretion of other tobacco constituents. A strong association was observed between the levels of urinary NNAL-*N*-Gluc and urinary nicotine-Gluc but not the *O*-glucuronidated metabolite of 3′-OH-cotinine in smokers in this study, a pattern consistent with the *N*-glucuronidating capacity of UGT2B10 previously described in *in vitro* studies (20, 23, 37).

The *UGT2B17* CNV was associated with the urinary levels of (*R*)-NNAL-*O*-Gluc but not (*S*)-NNAL-*O*-Gluc in smokers in this study, a pattern consistent with recent *in vitro* studies demonstrating that UGT2B17 prefers (*R*)-NNAL over (*S*)-NNAL as a substrate (26). These data are also consistent with previous *in vitro* studies demonstrating that the *UGT2B17* deletion was associated with decreases in total NNAL-*O*-Gluc formation (24) and, more recently, with (*R*)-NNAL-*O*-Gluc formation (26) in HLM. The fact that the *UGT2B17* deletion was associated with a decrease of 27% in the urinary ratio of (*R*)-NNAL-*O*-Gluc/(*S*)-NNAL-*O*-Gluc suggests that other UGTs, presumably UGTs 2B7 and 1A9, may also play important roles in the excretion of (*R*)-NNAL *in vivo*. This is consistent with the fact that these enzymes are all hepatically well expressed (38). It is also interesting that despite the relatively low percentage of urinary (*R*)-NNAL-*O*-Gluc associated with the *UGT2B17* deletion in smokers, a significant correlation was observed between the *UGT2B17* deletion and increased risk for lung adenocarcinoma in women (27). This is likely because of the fact that UGT2B17 is relatively well expressed in lung, a pattern not observed for other NNAL-glucuronidating enzymes including UGTs 2B7, 1A9, or 2B10 (39, 40). To better examine this, studies

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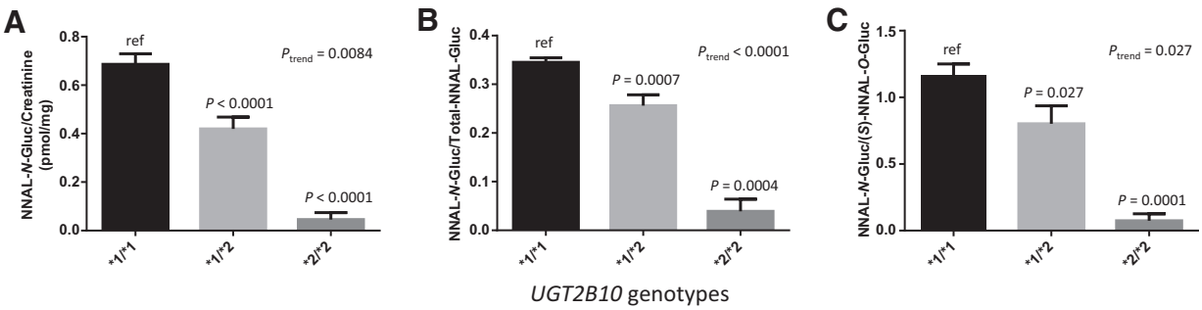

Figure 3.

Association between the *UGT2B10* codon 67 polymorphism and urinary NNAL-*N*-Gluc levels in smokers. Subjects were stratified by *UGT2B10* codon 67 (Asp>Tyr) genotype, with the *UGT2B10*1* allele corresponding to the wild-type UGT2B10^{67Asp} and the *UGT2B10*2* allele corresponding to the UGT2B10^{67Tyr} variant. A, *UGT2B10* genotypes versus creatinine-adjusted NNAL-*N*-Gluc (pmol/mg creatinine); B, *UGT2B10* genotypes versus the ratio of NNAL-*N*-Gluc/total-NNAL-Gluc; and C, *UGT2B10* genotypes versus the ratio of NNAL-*N*-Gluc/(*S*)-NNAL-*O*-Gluc. All values are expressed as the mean ± SEM for 174 genotype-informative subjects. For comparative analysis between genotypes, the Student *t* test was performed on squared-root transformed data for panel A only; nontransformed data were used for panels B and C.

are currently underway attempting to examine the relationship between the *UGT2B17* deletion genotype and NNAL glucuronidating activities in human lung microsomes.

A limitation of this study is the fact that all of the urine specimens examined in this study were from Caucasian subjects. It would be interesting to expand to incorporate subjects from other racial or ethnic groups. For instance, a functional splice site SNP in UGT2B10 (rs116294140) is relatively prevalent in African Americans but was not incorporated into the present analysis because of its low prevalence (<1%) in Caucasians (41). Another limitation is that dietary information was not collected from the individual subjects examined in this study. Therefore, the potential influence of other factors including specific dietary exposures on NNAL metabolism could not be performed in this study.

The average urinary levels of NNAL and its metabolites in smokers were below 1 nmol/L in this study. This is more than 10,000-fold lower than the levels of urinary nicotine and its major metabolite, cotinine (42, 43). It is, therefore, a technical challenge to directly quantify NNAL and its metabolites in the urine of tobacco users. Previous studies from the Hecht lab (University of Minnesota) have examined urinary NNAL and its glucuronides by measuring, (i) free NNAL, (ii) free NNAL + NNAL-*N*-Gluc, and (iii) total-NNAL, with the levels of both NNAL-*N*-Gluc and total NNAL-*O*-Gluc mathematically derived. This methodology incorporates several sample processing steps and corresponding MS runs using up to 180 μL of urine specimen and a nano-LC/MS instrument (35). This method is sensitive, reaching levels as low as 0.10 nmol/L for the quantification of urinary-free NNAL (35, 44). Limitations of this procedure include the fact that NNAL-*N*-Gluc and NNAL-*O*-Gluc are not directly measured, and that the (*R*)- and (*S*)-NNAL-*O*-Gluc diasteriomers are not measured individually. A LC/MS method for the simultaneous quantification of intact NNAL-*O*-Gluc and NNAL-*N*-Gluc (and free NNAL) was recently described by Yao and colleagues (45). This method is highly sensitive, reaching detection levels as low as 0.007, 0.039, and 0.055 nmol/L for free NNAL, NNAL-N-Gluc, and NNAL-O-Gluc, respectively, and incorporates a purification step using dichloromethane and a further extraction step by solid-phase extraction which concentrates each urine sample 100-fold. The (*R*)- and (*S*)-NNAL-*O*-Gluc diastereomers are not measured individually within this methodology.

The LC/MS method developed in the present study is the first to simultaneously and directly quantify the urinary levels of free NNAL, NNAL-*N*-Gluc, and the individual (*R*)-NNAL-*O*-Gluc and (*S*)-NNAL-*O*-Gluc diastereomers in tobacco users. It requires only 10 μL of urine sample, no sample incubation or extraction steps, and a single LC/MS analysis. The average percentage of NNAL-*N*-Gluc, NNAL-*O*-Gluc [(*R*)-NNAL-*O*-Gluc + (*S*)-NNAL-*O*-Gluc], and free-NNAL in the urine samples from smokers in this study were 23%, 49%, and 28%, respectively, which is highly consistent with that observed in smokers in previous studies (22%, 48% and 31%, respectively; ref. 35). These data suggest that the values obtained using the methodology described in this study are highly accurate. Although the lower limit of detection for urinary free NNAL in this study is similar to those of Hecht's studies (35), it is approximately 14-fold higher than the detection limits observed for NNAL using the Yao and colleagues methodology (45). However, the limits of detection observed in the present study for NNAL-*N*-Gluc (0.10 nmol/L) and each of the NNAL-O-Gluc diastereomers [0.025 nmol/L for (*S*)-NNAL-*O*-Gluc and 0.050 nmol/L for (*R*)-NNAL-*O*-Gluc] are similar to that observed for NNAL-*N*-Gluc (0.039 nmol/L) and total NNAL-*O*-Gluc (0.055 nmol/L) in the Yao and colleagues methodology (45). In addition, the lower limits of detection observed for urinary NNAL-*N*-Gluc, (*R*)-NNAL-O-Gluc, and (*S*)-NNAL-*O*-Gluc are similar to or lower than that observed for free NNAL (0.1 nmol/L). Together, these data suggest that the methodology described in the present study is quite sensitive for the direct detection of these glucuronidated NNAL metabolites.

It has been found that (*S*)-NNAL is stereoselectively retained in rat lung and has a higher tumorigenicity than (*R*)-NNAL, and (*R*)-NNAL exhibits a higher rate of glucuronidation in rats (17, 18, 46) and the A/J mouse (47, 48). Data from previous studies suggest that (*S*)-NNAL may also be stereoselectively retained in humans (19). However, the levels of urinary (*S*)-NNAL-*O*-Gluc were higher than the levels of urinary (*R*)-NNAL-*O*-Gluc in smokers (19), tobacco chewers (19), and in the patas monkey (49), a pattern consistent with the results from the present study where the mean levels of urinary (*S*)-NNAL-*O*-Gluc was approximately

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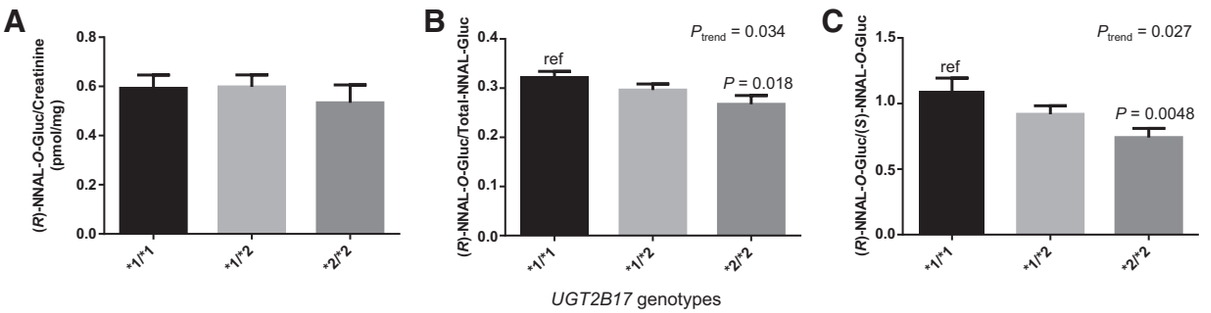

Figure 4.

Association between the *UGT2B17* deletion polymorphism and urinary (*R*)-NNAL-*O*-Gluc levels in smokers. Subjects were stratified by *UGT2B17* CNV genotypes, with the *UGT2B17**1 allele corresponding to the wild-type single-gene copy number and the *UGT2B17**2 allele corresponding to the *UGT2B17* gene deletion variant. A, *UGT2B17* CNV genotypes versus creatinine-adjusted (*R*)-NNAL-*O*-Gluc (pmol/mg creatinine); B, *UGT2B17* CNV genotypes versus the ratio of (*R*)-NNAL-*O*-Gluc/total-NNAL-Gluc; and C, *UGT2B17* CNV genotypes versus the ratio of (*R*)-NNAL-*O*-Gluc/(*S*)-NNAL-*O*-Gluc. All values are expressed as the mean ± SEM for 180 genotype-informative subjects.

25% higher than the mean levels of urinary (*R*)-NNAL-O-Gluc in smokers. These data could reflect higher levels of (S)-NNAL formed from NNK during NNK metabolism after ingestion/inhalation, or be due to a preferential glucuronidation of (*S*)-NNAL, in primates versus rodents. Further studies will be required to examine this in full.

As indicated in Table 2, there were individual values for NNAL metabolites that were below the detection limit; these raw values were used as true values for this study. To confirm the UGT genotype: urinary NNAL metabolite phenotype associations observed in the present study, we also applied another strategy in dealing with values that are below the limit of detection by arbitrarily setting any nondetectable value equal to one-half of the minimum level of detection for that metabolite [0.05 nmol/L for NNAL-*N*-Gluc, 0.025 nmol/L for (*R*)-NNAL-*O*-Gluc, 0.0125 nmol/L for (*S*)-NNAL-*O*-Gluc, and 0.05 nmol/L for free NNAL]. Virtually identical statistically significant results were observed for all associations using this alternate strategy (results not shown).

In summary, NNAL-*N*-Gluc, (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, and free NNAL in smokers' urine were simultaneously and directly determined using a simple LC/MS analysis to demonstrate, for the first time, strong genotype–phenotype associations between both the *UGT2B10* codon 67 (Asp>Tyr) genotype and urinary NNAL-*N*-Gluc levels, and the *UGT2B17* CNV and urinary (*R*)-NNAL-*O*-Gluc levels, in smokers. These results suggest that functional polymorphisms in *UGT2B10* and *UGT2B17* are associated with reduced detoxification capacity against NNAL and therefore may affect individual cancer risk upon tobacco exposure.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G. Chen, P. Lazarus
Development of methodology: G. Chen, S. Luo, S. Kozlovich, P. Lazarus
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Chen, S. Luo, P. Lazarus
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Chen, S. Luo, P. Lazarus
Writing, review, and/or revision of the manuscript: G. Chen, S. Luo, S. Kozlovich, P. Lazarus
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Lazarus
Study supervision: P. Lazarus

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