Toxicology Letters 216 (2013) 16–22

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) metabolism-related enzymes gene polymorphisms, NNK metabolites levels and urothelial carcinoma

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HIGHLIGHTS

- Significant dose–response associations between NNK-related metabolites and UC risk.
- Subjects with the UGT2B7 codon 268 His/Tyr or Tyr/Tyr genotype had lower total NNAL.
- No association was seen between gene polymorphisms of CYP2A13 and UGT2B7 and UC risk.

ARTICLE INFO

Article history:
Received 21 August 2012
Received in revised form 1 November 2012
Accepted 2 November 2012
Available online 8 November 2012

Keywords:
NNK
CYP2A13
UGT2B7
Polymorphism
NNAL
Urothelial carcinoma

ABSTRACT

Gene polymorphisms of the 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) metabolism-related enzymes-cytochrome P450 (CYP) monooxygenase 2A13 (CYP2A13) and UDP-glucuronosyltransferases (UGT)-2B7 could contribute to the levels of NNK-related metabolites in urine, thereby increasing the susceptibility to urothelial carcinoma (UC). Therefore, our study aimed to evaluate the roles of two gene polymorphisms (CYP2A13 and UGT2B7) of NNK metabolism-related enzymes in the carcinogenesis of UC in Taiwan. A hospital-based pilot case–control study was conducted. There were 121 UC cases and 121 age- and sex-matched healthy participants recruited from March 2007 to April 2009. Urine samples were analyzed for NNK-related metabolites using the liquid chromatography–tandem mass spectrometry method. Genotyping was conducted using a polymerase chain reaction-restriction fragment length polymorphism technique. ANCOVA and multivariate logistic regression were applied for data analyses. In healthy controls, former smokers had significantly higher total NNAL and higher NNAL-Gluc than never smokers or current smokers. Subjects carrying the *UGT2B7* 268 His/Tyr or Tyr/Tyr genotype had significantly lower total NNAL than those carrying His/His genotype. However, no association was seen between gene polymorphisms of CYP2A13 and UGT2B7 and UC risk after adjustment for age and sex. Significant dose -response associations between total NNAL, free NNAL, the ratios of free NNAL/total NNAL and NNAL-Gluc/total NNAL and UC risk were observed. In the future, large-scale studies will be required to verify the association between the single nucleotide polymorphisms of NNK metabolism-related enzymes and UC risk.

1. Introduction

Urothelial carcinoma (UC) originates exclusively from the urothelium present throughout the urinary tract, including the renal pelvis, ureter, bladder, and urethra. Of the UCs, bladder cancer is the most predominant type. The most important risk factor of

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http://dx.doi.org/10.1016/j.toxlet.2012.11.002

bladder cancer is cigarette smoking, containing more than 60 carcinogenic compounds, which might increase proliferation of the bladder epithelium and induce tumorgenesis (Strope and Montie, 2008; Zaridze et al., 1991).

The compound 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine-derived, tobacco-specific nitrosamine, 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 carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) to exert its carcinogenic effects (Richter et al., 2009). NNAL itself is a carcinogen and is further detoxified to either O- or N-glucuronide, known as NNAL-glucuronides (NNAL-Gluc) (Tricker et al., 2001). These NNK-related metabolites are ultimately excreted in urine. The carcinogenic activity of NNK in the lung has been repeatedly shown in various animal studies, regardless of the route of administration or even with low-dose exposure (Hecht et al., 1999; Rivenson et al., 1988). In human studies, NNK-related metabolites were useful biomarkers in smokers and were found in cancer tissue of patients with bladder cancer (Carmella et al., 2005; Saad et al., 2006).

The polymorphisms of enzymes affecting NNK-related metabolism have been found in cytochrome P450 (CYP) monooxygenase 2A13 (CYP2A13) and in UDP-glucuronosyltransferases (UGT), including CYP2A13 Arg257Cys and UGT2B7 His268Tyr (Zhang et al., 2002). CYP2A13 plays a more important role than any other human P450 enzyme, having a high catalytic efficiency to activate NNK and NNAL by alpha-hydroxylation (Jalas et al., 2005). UGT2B7 belongs to the UGT superfamily of enzymes, which perform the glucuronidation of xenobiotics and endogenous compounds and may catalyze the O-glucuronidation of NNAL (Ren et al., 2000). The formation of NNAL-Gluc was an important product in the detoxification of NNAL and NNK (Wiener et al., 2004). Studies indicate that genetic variations of CYP2A13 Arg257Cys and UGT2B7 His268Tyr were associated with altered enzymatic activity (Wiener et al., 2004; Zhang et al., 2002). Epidemiologic studies have found conflicting associations between gene polymorphisms of CYP2A13 Arg257Cys, as well as UGT2B7 His268Tyr, and various cancers, including bladder, colorectal, head and neck, and lung (D'Agostino et al., 2008; Sharma et al., 2010; van der Logt et al., 2009; Zimmermann et al., 2008). Previously, our studies found that the levels of urinary total NNAL (free NNAL plus NNAL-Gluc) were positively correlated with cigarette smoking status; the ratios of free NNAL/total NNAL or NNAL-Gluc/total NNAL were useful biomarkers for UC (Chung et al., 2011; Lee et al., 2008). However, the relationship between these genetic variants, urinary NNK-related metabolites, and UC risk has seldom been investigated. Therefore, we conducted a pilot study to evaluate whether the gene polymorphisms of CYP2A13 and UGT2B7 modified risk of UC by affecting urinary NNK-related metabolites.

2. Materials and methods

2.1. Study participants

A hospital-based, case–control study was conducted. The study design has been described previously (Pu et al., 2007). Briefly, from March 2007 to April 2009, 121 UC cases and 121 age- and sex-matched healthy participants as controls were recruited from the Medical Center including the National Taiwan University Hospital and the Taipei Municipal Wan Fang Hospital. All UC cases were diagnosed by histological confirmation, and none presented with other histology, such as squamous cell carcinoma, adenocarcinoma, sarcoma, lymphoma, or benign lesions. Healthy controls with no prior history of cancer were matched to UC cases by age and sex. All study subjects provided informed consent prior to a questionnaire interview and biological specimen collection. The Research Ethics Committee of the National Taiwan University Hospital, Taipei, Taiwan, approved the study, and it was conducted in agreement with the World Medical Association Declaration of Helsinki.

2.2. Questionnaire interview

Well-trained interviewers collected information on lifestyle risk factors using a structured questionnaire administered during a face-to-face interview. The information collected included demographics and socioeconomic characteristics, lifestyle factors (such as cigarette smoking, environmental tobacco smoke; ETS), and personal and family medical history.

2.3. Biological specimen collection

Spot urine samples (50 mL) were collected at the time of recruitment and immediately transferred to a −20 °C freezer until needed for analysis of urinary NNK-related metabolites. Concurrently, peripheral blood samples (5–8 mL) were collected using ethylene-diamine-tetraacetic acid (EDTA) vacuumed syringes, and the separated buffy coat was frozen at −80 °C for DNA extraction and identification of enzyme gene polymorphisms.

2.4. Urinary analysis of NNK-related metabolites

Analysis of urinary NNK-related metabolite levels was done using a liquid chromatography–tandem mass spectrometry; the protocol has been described previously (Lee et al., 2007). Recovery rates for NNAL were approximately 99%, with detection limits ranging from 0.2 to 2.4 pg and the quantity of samples injected ranging from 0.01 to 0.12 ng/mL. The average intra- and inter-day variations of repeated measurements in five samples were 6.2% and 5.5%, respectively.

2.5. Genotyping

Genomic DNA was extracted using proteinase K digestion following phenol and chloroform extraction. Genotyping for single nucleotide polymorphisms (SNPs) in CYP2A13 Arg257Cys (C3375T) and UGT2B7 His268Tyr (C802T) was performed by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (Wang et al., 2003; Wiener et al., 2004). Briefly, the following primers were used to amplify 332 bp and 116 bp PCR products for CYP2A13 and UGT2B7, respectively: 5′-CCTGGACAGATGCCTTTAACTCCG-3′ (forward) and 5′-TGGCTTTGCACCTGCCTGCACT-3′ (backward) for CYP2A13; and 5′-GACAATGGGGAAAGCTGACG-3′ (forward) and 5′-GTTTGGCAGGTTTGCAGTG-3′ (backward) for UGT2B7. PCR products were obtained in a total volume of 30 μL, consisting of an 80 ng sample DNA, 10× PCR buffer, 2.5 mM dNTP, 2 μM of each primer, and 2 U Taq polymerase. After initial denaturation for 3 min at 94 °C, 35 cycles were performed at 95 °C for 30 s (denaturation), at 63 °C for 45 s (annealing), and a 72 °C for 30 s (extension) for CYP2A13. For UGT2B7, the cycles were performed at 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, followed by a final step at 72 °C for 5 min. The amplified products were visualized by electrophoresis in a 2% agarose gel. PCR products were digested with HhaI (>3 h at 37 °C) for CYP2A13, and BseGI (>3 h at 37 °C) for UGT2B7. The products were then analyzed again by electrophoresis on 3% agarose gels. A random 5% of the samples were repeated with a concordance of 100% for quality control.

2.6. Statistical analysis

Hardy–Weinberg equilibrium was calculated by the goodness of fit χ^2 test, and the distributions of CYP2A13 and UGT2B7 genotypes fit the Hardy–Weinberg equilibrium. Cigarette smoking status included never, former, and current. We defined current smokers as those who were still smoking at the time of the interview and former smokers as those who had quit smoking at the time of recruitment. Never smokers reported no smoking in their lifetimes. Information on the duration (years) and frequency (packs per day) of smoking habits were also collected to calculate the cumulative exposure of cigarette smoking (pack-years). Urinary total NNAL (ng/mL) was defined as the sum of free NNAL and NNAL-Gluc. All metabolites of total NNAL, free NNAL, and NNAL-Gluc were normalized by urinary creatinine (μg/g creatinine or mg/g creatinine). The ratios of free NNAL/total NNAL and NNAL-Gluc/total NNAL were calculated by dividing the concentration of each species (free NNAL or NNAL-Gluc) by the total NNAL concentration. ANCOVA analysis was used to compare the differences in related risk factors between UC cases and controls after adjusting for age, gender, and other risk factors. In addition, multivariate logistic regression models were used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) to determine the association between genotypes of CYP2A13 and UGT2B7 and the risk of UC after adjustment for age, sex, educational level, and smoking status. The cutoff values for NNK-related metabolites were the medians or tertiles of the controls in the interaction analysis or in the dose–response relationships, respectively. All analyses were conducted using Statistical Analysis Software (SAS) statistical package (SAS, version 8.0, Cary, NC, USA).

3. Results

The distributions of sociodemographic characteristics and smoking status, as well as CYP2A13 and UGT2B7 genotypes, are shown in Table 1. Healthy controls had higher educational levels