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Modification of urinary N7-methylguanine excretion in smokers by glutathione-*S*-transferase M1 polymorphism

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ABSTRACT

Tobacco smoke contains many carcinogens which can lead to DNA methylation. Glutathione-*S*-transferases (GSTs) are detoxifying enzymes, and the effects of the genes *GSTM1*, *GSTT1*, and *GSTP1* on cigarette smoke-induced methylated adducts have not been elucidated. Therefore, we investigated the association of the *GSTM1*, *GSTT1*, and *GSTP1* genes and N7-methylguanine (N7-MeG) adducts in smokers. Urinary N7-MeG concentrations were measured by using liquid chromatography–tandem mass spectrometry in 112 smokers and 89 non-smokers, and *GSTM1*, *GSTT1*, and *GSTP1* genotypes were identified by polymerase chain reaction. Smokers had higher N7-MeG concentrations than did non-smokers (3238 ± 305 ng/mg creatinine [standard error] vs. 2386 ± 153 ng/mg creatinine; $P = 0.01$). Higher N7-MeG concentrations were observed with the *GSTM1* null genotype than with the *GSTM1* non-null genotype (3230 ± 292 ng/mg creatinine vs. 2336 ± 153 ng/mg creatinine; $P = 0.007$), particularly in smokers (3775 ± 483 ng/mg creatinine vs. 2468 ± 228 ng/mg creatinine; $P = 0.02$). However, the *GSTT1* and *GSTP1* genotypes were not associated with urinary N7-MeG concentrations. Therefore, the susceptible *GSTM1* genotype may modulate the concentrations of N7-MeG adducts in the DNA of smokers.

1. Introduction

Tobacco smoke contains many carcinogens known to bind covalently to DNA (Hemminki, 1983). The metabolic activation of tobacco smoke carcinogens in target tissues results in the formation of methylated DNA bases (Hecht, 1999a) which may partially contribute to the elevated lung cancer incidence in smokers (Hecht, 1999b; Loft et al., 2007). Alkylating agents react predominantly with the N7 position of guanine (Haque et al., 1997). N7-methylguanine (N7-MeG) lesions have been used as markers of exposure to *N*-nitroso compounds, in part because of their relatively high concentrations in DNA and also because their repair rate is much slower than that of other pro-mutagenic and carcinogenic lesions, such as O^6-alkylguanine (van Delft et al., 1992). Whereas N7-MeG is innocuous in DNA, it may reflect the overall rate of DNA methylation and correlate with the formation of pro-mutagenic and carcinogenic methyl-adducts, such as O^6-methylguanine and methyladenine (Chiang et al., 2005; Meer et al., 1986; Van Benthem et al., 1994). Thus, N7-MeG may serve as a biomarker of exposure to methylating agents.

Many carcinogen–DNA adducts can induce mutations and are formed from the reaction of carcinogen metabolites and DNA. They represent the net effects of mutagen exposure, absorption, activation, detoxification, and DNA repair. Glutathione-*S*-transferases (GSTs) constitute a superfamily of phase II enzymes that detoxify carcinogens, including those from tobacco smoke. GSTs protect against DNA damage by glutathione conjugation to reactive metabolic species that can form protein or DNA adducts and generate reactive oxygen species (Hayes and Strange, 1995). Metabolic polymorphisms have been implicated in chemical exposure-related health effects (Chen et al., 1996; Jourenkova-Mironova et al., 1998). The *GSTM1* (Bell et al., 1992; Chen et al., 1996), *GSTT1* (Chen et al., 1996), and *GSTP1* (Watson et al., 1998) genes are polymorphic. Some studies observed that DNA adduct concentrations were higher among *GSTM1*-null and *GSTT1*-null individuals (Ketelslegers et al., 2006; Lodovici et al., 2004; Peluso et al., 2004), although such associations are not consistently found (Cheng et al., 2000; Saad et al., 2006).

Therefore, smokers with inherited *GSTM1*, *GSTT1*, and *GSTP1* metabolic genotypes that affect susceptibility to DNA damage may have elevated urinary N7-MeG concentrations. We designed a

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population-based, cross-sectional study to investigate the association of metabolic genetic polymorphisms and urinary N7-MeG concentrations in smokers.

2. Materials and methods

2.1. Study subjects and biospecimen collection

A community-based health examination was performed in Nantou in central Taiwan from May 2006 to February 2007. The study protocol conformed to the Declaration of Helsinki and was approved by relevant ethics committees from participating institutions. Local residents older than 18 years of age were invited to participate, and 112 smokers and 89 non-smokers were recruited into the study. Study participants were randomly selected from a pool of eligible subjects, and the selected participants responded to detailed questioning, were willing to provide biospecimens, and were cancer-free. We also attempted to minimize possible selection bias by recruiting subjects from the same geographic areas, with the same ethnicities, and similar socioeconomic status.

Personal information and characteristics were collected from the study subjects by using interviewer-administered questionnaires during the medical surveillance phase of the study. Informed consent was obtained from all participants. The structured questionnaire contained questions involving demographic characteristics and lifestyle (including cigarette smoking and alcohol intake). The subjects answered questions discussing the amount, frequency, and duration of smoking. Habitual alcohol drinking was defined as alcohol consumption at least once weekly and of more than 80 g of alcohol weekly, as previously described (Wong et al., 2008). Urine and venous blood samples were collected in the morning of a weekday, stored at 4 °C, and processed on the same day. The blood was centrifuged to separate the serum and the cells. All specimens were stored at −70 °C conditions until analyzed.

2.2. Measurement of urinary N7-MeG concentrations

Urinary N7-MeG concentrations were measured as previously described by Chao et al. (2005) using isotope-dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS). Briefly, urine samples were thawed and thoroughly mixed at room temperature. Samples were centrifuged at 10,000 × *g* for 5 min and then urine (50 μL) was diluted 1000-fold with 96% acetonitrile containing 0.1% formic acid. To the diluted urine, we added 100 μL of $^{15}N_5$-N7-MeG solution (0.62 ng/mL) as internal standard followed by vigorous vortexing.

Prepared urine (100 μL) was then analyzed by LC–MS/MS coupled with an online enrichment system with an automatic column-switching device, which was previously described (Liao et al., 2002). In brief, the column-switching device has a switching valve (two-position microelectric actuator; Valco, Houston, TX, USA) and a Nucleosil NH_2 cartridge [35 mm × 4.6 mm inner diameter (i.d.), 10 μm]. The total run time was 15 min. The high-performance liquid chromatography (HPLC) system consisted of 2 series 200 micropumps, a series 200 autosampler (PerkinElmer, Boston, MA, USA), and a polyamine-II endcapped HPLC column (150 mm × 4.6 mm, 5 μm bead size; YMC Inc., Wilmington, NC, USA) with a guard column (10 mm × 2 mm; YMC Inc.). A gradient program was used to separate the analytes. The mobile phase was 90% acetonitrile containing 0.1% formic acid and was delivered at a flow rate of 1 mL/min for 3 min. The phase was then varied to 80% acetonitrile with 0.1% formic acid within 10 min and was rapidly delivered to the previous 90% acetonitrile solvent containing 0.1% formic acid with a linear gradient in 1 min. The HPLC system elution was introduced into a TurboionSpray source installed on a triple–quadrupole mass spectrometer (API 3000; Applied Biosystems, Foster City, CA, USA) and was operated in the positive-ion mode. The limit of detection of the method was 4.8 fmol. Urinary creatinine concentrations were used to correct for variations in urine concentrations in this study. The creatinine concentration in the urine sample was measured with a Shimadzu model UV-1601 spectrophotometer using the Jaffé reaction (Jaffé, 1886).

2.3. Polymorphisms of GSTM1, GSTT1, and GSTP1 genes

Genomic DNA was extracted from the peripheral blood of subjects using the QIAmp blood kit (Qiagen, Chatsworth, CA, USA). The *GSTM1* and *GSTT1* genotypes were determined by co-amplification of the two genes (Liu et al., 2006). Primers used in the polymerase chain reaction (PCR) for the *GSTM1* gene were 5′-CTG CCC TAC TTG ATT GAT GGG-3′ and 5′-CTG GAT TGT AGC AGA TCA TGC-3′. The primers used for the *GSTT1* gene were 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′ and 5′-TCA CCG GAT CAT GGC CAG CA-3′. Human *β-globin* (110 bp) was also amplified as a positive control in each reaction with the primers 5′-ACA CAA CTG TGT TCA CTA GC-3′ and 5′-CAA CTT CAT CCA CGT TCA CC-3′. The reaction mixture consisted of a 0.5-μL sample of DNA and a PCR buffer containing 200 ng of the following primers: 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 50 mM of KCl, 10 mM of Tris–HCl (pH 8.3), and 0.1% of bovine serum albumin to a final volume of 50 μL. Reaction mixtures were heated for 5 min at 94 °C. A total of 35 amplification cycles were run as follows: a denaturing step at 94 °C for 1 min 30 s, an annealing step at 59 °C for 1 min, and an extension step at 72 °C for 1 min. Reaction products were run on a 2% agarose gel. Individuals with one or more *GSTM1* alleles had a 273-bp fragment, and individuals with one or more *GSTT1* alleles had a 480-bp fragment.

The *GSTP1* polymorphism was also determined using a PCR-restriction fragment length polymorphism (RFLP) technique (Liu et al., 2006). An *Ile* to *Val* substitution in exon 5 (codon 105) was amplified to form an undigested 177 bp fragment using the primer pair 5′-ACC CCA GGG CTC TAT GGG AA-3′and 5′-TGA GGG CAC AAG AAG CCC CT-3′. The amplification cycles were run as follows: a denaturing step at 94 °C for 30 s, an annealing step at 61 °C for 30 s, and an extension step at 72 °C for 30 s. The PCR products were digested with *Alw*26I. Homozygous *Ile-Ile* individuals had a single fragment of 177 bp, homozygous *Val-Val* individuals had both 92- and 85-bp fragments, and heterozygous *Ile-Val* individuals had all three fragments.

2.4. Statistical analysis

Smokers and non-smokers were compared for differences in age at recruitment, sex, years of education, and alcohol drinking using the Student's *t* test for continuous variables and the chi-square test for discrete variables. The χ^2-test was used to test the significance of differences in prevalence of the *GSTM1*, *GSTT1*, and *GSTP1* genotypes between smokers and non-smokers. Crude urinary N7-MeG concentrations were evaluated using analyses stratified by smoking status and various factors. Student's *t* test and analysis of variance (ANOVA) were used to examine differences in urinary N7-MeG concentrations by smoking status, age, sex, years of education, alcohol drinking, and metabolic traits. Tests for differences in least squares means were calculated to predict adjusted urinary N7-MeG concentrations for our study subjects stratified by different smoking status and metabolic genotypes. All *P* values were calculated using two-tailed statistical tests, and statistical significance was defined at *P* values < 0.05. All data were analyzed using SAS 9.1 (SAS Institute, Cary, NC, USA).

3. Results

Basic characteristics of the study participants are summarized in Table 1. A statistically significant difference in age distribution was identified between smokers and non-smokers ($P = 0.048$, χ^2-test). The mean age at study recruitment was 44.9 years (range: 19–87 years) for smokers and 46.6 years (range: 18–71 years) for non-smokers ($P = 0.37$, *t* test). There were no differences in sex ($P = 0.09$) or educational level ($P = 0.82$) between smokers and non-smokers. Approximately half of all smokers smoked more than 20 pack-years (49.1%). Alcohol drinking was significantly greater in smokers than in non-smokers (78.6% vs. 37.1%; $P < 0.001$). The prevalence of *GSTM1* ($P = 0.94$), *GSTT1* ($P = 0.28$), and *GSTP1* ($P = 0.60$) genotypes were not significantly different between smokers and non-smokers.

The crude associations of urinary N7-MeG concentrations with various factors are summarized in Table 2 for all tested individuals. Compared with non-smokers, smokers had significantly higher urinary N7-MeG concentrations (3238 ng/mg creatinine vs. 2386 ng/mg creatinine; $P = 0.01$). Individuals with a median age of 45 years or older had higher urinary N7-MeG concentrations than did individuals younger than 45 years, particularly non-smokers (2648 ng/mg creatinine vs. 1962 ng/mg creatinine; $P = 0.02$). Individuals with fewer than 6 years of formal education had higher urinary N7-MeG concentrations (3395 ng/mg creatinine) than did individuals with more than 6 years of formal education (7–12 years: 2357 ng/mg creatinine; >12 years: 2919 ng/mg creatinine), particularly in smokers (4041 ng/mg creatinine vs. 2529, 3128 ng/mg creatinine; $P = 0.07$). Sex and alcohol drinking were not associated with elevated urinary N7-MeG concentrations. Higher urinary N7-MeG concentrations were observed in individuals with the *GSTM1* null genotype than in individuals with the *GSTM1* non-null genotype (3230 ng/mg creatinine vs. 2336 ng/mg creatinine; $P = 0.007$), particularly in smokers (3775 ng/mg creatinine vs. 2468 ng/mg creatinine; $P = 0.02$). No significant associations were observed between urinary N7-MeG concentrations and *GSTT1* and *GSTP1* genotypes.

Years of education and age were associated with crude urinary N7-MeG concentrations in the univariate analysis. Smokers and non-smokers also had different age and alcohol drinking distributions. Therefore, a least squares mean analysis was performed to assess the joint effect of the *GSTM1* and smoking status on urinary N7-MeG concentrations. We adjusted for the effects of age, years of

Table 1
Frequency distribution of select characteristics stratified by smoking status.

Variable	Non-smokers (N = 89)	Smokers (N = 112)
Age (years)	46.6 ± 1.3	44.9 ± 1.4
≥60 years	12 (13.5%)	21 (18.8%)*
50–59 years	25 (28.1%)	16 (14.3%)
<50 years	52 (58.4%)	75 (67.0%)
Sex		
Male	72 (80.9%)	100 (89.3%)
Female	17 (19.1%)	12 (10.7%)
Years of education		
0–6	37 (41.6%)	45 (40.2%)
7–12	44 (49.4%)	54 (48.2%)
>12	8 (10.0%)	13 (11.6%)
Smoking dose		
>20 cigarettes/day		21 (18.8%)
11–20 cigarettes/day		55 (49.1%)
1–10 cigarettes/day		36 (32.1%)
Pack-years smoked		
>20		55 (49.1%)
11–20		29 (25.9%)
1–10		28 (25.0%)
Alcohol drinking		
Yes	33 (37.1%)	88 (78.6%)**
No	56 (62.9%)	24 (21.4%)
GSTM1 genotype		
Null	52 (58.4%)	66 (58.9%)
Non-null	37 (41.6%)	46 (41.1%)
GSTT1 genotype		
Null	44 (49.4%)	64 (57.1%)
Non-null	45 (50.6%)	48 (42.9%)
GSTP1 genotype		
Ile-Ile	36 (40.5%)	40 (35.7%)
Ile-Val	20 (22.5%)	32 (28.6%)
Val-Val	33 (37.0%)	40 (35.7%)

Data are reported as the number of individuals and percentages or means ± standard errors. A two-sided χ^2-test was used to test differences between discrete variables and a t test was used for continuous variables.

* P = 0.05 compared with the non-smokers group.

** P < 0.01 compared with the non-smokers group.

Table 2
Crude urinary N7-methylguanine (ng/mg creatinine) concentrations in smokers and non-smokers stratified by various factors.

	Non-smokers		Smokers		All	
	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
All	89	2386 ± 153	112	3238 ± 305[a]	201	2861 ± 185
Age						
≥45 years (median)	55	2648 ± 211[b]	46	3733 ± 610	101	3142 ± 304
<45 years	34	1962 ± 190	66	2893 ± 292	100	2577 ± 207
Sex						
Males	72	2366 ± 159	100	3199 ± 325	172	2850 ± 202
Females	17	2470 ± 440	12	3567 ± 894	29	2924 ± 453
Years of education						
0–6	37	2628 ± 240	45	4041 ± 642	82	3395 ± 372[c]
7–12	44	2147 ± 210	54	2529 ± 285	98	2357 ± 183
>12	8	2580 ± 561	13	3128 ± 650	21	2919 ± 450
Alcohol drinking						
Yes	33	2620 ± 288	88	3185 ± 362	121	3031 ± 275
No	56	2247 ± 173	24	3436 ± 519	80	2604 ± 205
GSTM1						
Null	52	2537 ± 220	66	3775 ± 483[d]	118	3230 ± 292[e]
Non-null	37	2173 ± 195	46	2468 ± 228	83	2336 ± 153
GSTT1						
Null	44	2441 ± 231	64	3412 ± 459	108	3021 ± 291
Non-null	45	2331 ± 202	48	3007 ± 364	93	2680 ± 214
GSTP1						
Ile-Ile	36	2660 ± 282	40	3545 ± 662	76	3126 ± 375
Ile-Val	20	1870 ± 179	32	3540 ± 525	52	2898 ± 348
Val-Val	33	2399 ± 242	40	2690 ± 336	73	2559 ± 214

[a] P = 0.01 compared with the non-smokers group (t test).

[b] P = 0.02 compared with the group aged <45 years (t test).

[c] P = 0.03 compared with the groups with 7–12 or >12 years of education (ANOVA).

[d] P = 0.02 by the t test, the *GSTM1* null genotype compared with the *GSTM1* non-null genotype in smokers.

[e] P = 0.007 compared with the *GSTM1* non-null genotype in all subjects (t test).

education, and alcohol drinking (Table 3). Individuals who smoked 1–10 cigarettes (the first quartile of smoking dose in smokers) per day had significantly higher urinary N7-MeG concentrations (3475 ng/mg creatinine) than did those in the no smoking category (2404 ng/mg creatinine; P = 0.048), whereas there was no dose–response relation between smoking dose and urinary N7-MeG concentrations. Individuals who smoked 1–10 cigarettes per day and had the *GSTM1* null genotype remained the highest urinary N7-MeG concentration (4317 ng/mg creatinine), and non-smokers with the *GSTM1* non-null genotype had the lowest urinary N7-MeG concentrations (2135 ng/mg creatinine). Study subjects who smoked greater than 10 cigarettes per day and had the *GSTM1* null genotype also had significantly higher urinary N7-MeG concentrations, compared to non-smokers with the *GSTM1* non-null genotype. Accumulated cigarette consumption was further stratified as no smoking, 1–10 pack-years smoked (the first quartile of cumulative dose in smokers), and greater than 10 pack-years smoked. The association between urinary N7-MeG concentrations and *GSTM1* was evaluated in this stratification scheme. Individuals with more pack-years of smoking had higher urinary N7-MeG concentrations than did those in the no smoking category, but this difference was not significant. However, a significant joint effect was observed between the susceptible *GSTM1* null genotype and

Table 3
Adjusted mean urinary N7-methylguanine concentrations (ng/mg creatinine) stratified by smoking status and the metabolic *GSTM1* genotypes.

Variables	Smoking dose (cigarettes/day)						Pack-years smoked					
	No		1–10		>10		0		1–10		>10	
	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
All	89	2404 ± 290 Reference	36	3475 ± 309 P = 0.048	76	3041 ± 309 P = 0.16	89	2404 ± 290 Reference	28	3346 ± 504 P = 0.12	84	3130 ± 295 P = 0.10
GSTM1												
Null	52	2560 ± 360 P = 0.43	19	4317 ± 599 P = 0.004	47	3477 ± 388 P = 0.03	52	2560 ± 360 P = 0.43	14	3848 ± 710 P = 0.05	52	3692 ± 370 P = 0.009
Non-null	37	2135 ± 435 Reference	17	2607 ± 618 P = 0.55	29	2356 ± 476 P = 0.76	37	2135 ± 435 Reference	14	2902 ± 683 P = 0.35	32	2263 ± 455 P = 0.84

Data were adjusted for the effects of age, years of education, and alcohol drinking.

the category of greater than 10 pack-years smoked (3692 ng/mg creatinine; $P = 0.009$) compared with the *GSTM1* non-null genotype and the no smoking category (2135 ng/mg creatinine).

4. Discussion

Humans are exposed to many kinds of carcinogens by smoking. The metabolic activation of these carcinogens may result in the formation of methylated DNA adducts (Hecht, 1999a). The N7-MeG is the most abundant base damaged in methylated DNA and is relatively easy to quantify. Therefore, N7-MeG may function as a superior biomarker for the assessment of human exposure to methylating agents (Chao et al., 2005; Haque et al., 1997; van Delft et al., 1992). The N7-MeG is well-recognized to spontaneously depurinate or to be depurinated as part of the pathway of base excision repair, and formation of an apurinic site is part of that process. Apurinic sites are effective mutagenic lesions if they are present during replication (Avkin et al., 2002; Gentil et al., 1992). Repair of N7-MeG in the body is poor, and systemic concentrations should reflect variation in exposure intensity and in metabolism (van Delft et al., 1992). Therefore, the formation of N7-MeG may partially contribute to lung cancer risk in smokers (Hecht, 1999b; Loft et al., 2007).

Previously, Bianchini et al. (1993) reported a detection limit of 0.5 pmol in the equivalent of 1 mg of DNA using an immunoaffinity purification and HPLC with electrochemical detection. Furthermore, Haque et al. (1997) used ^{32}P-postlabeling assays for quantitation of N7-MeG adducts in human DNA and obtained a 1.3-fmol detection limit. The ^{32}P-postlabeling approach, despite being time-consuming and requiring large amounts of radioactivity, has the advantage of having high sensitivity. A highly specific and sensitive LC–MS/MS method for the detection of N7-MeG in human urine samples has been established (Chao et al., 2005) and applied in the present study. With the use of isotopic internal standards and on-line solid-phase extraction system, this method had a comparable LOD of 4.8 fmol on column, and more importantly allowed us to directly measure the trace N7-MeG concentrations in the complex urine matrix without any prior purification.

Loft et al. (2007) investigated the elevated urinary excretion of methylated DNA adducts in a Danish cohort using an HPLC/ultraviolet method. They indicated that a higher urinary N7-MeG concentration was significantly associated with smoking status, whereas there was no dose–response relation between smoking and N7-MeG excretion. In the present study, we used a highly sensitive LC–MS/MS method to detect N7-MeG and observed that cancer-free smokers had higher urinary N7-MeG concentrations than did non-smokers. This suggested that elevated DNA methylation was the result of cigarette smoke being the main exposure source in our study participants. However, there was no significant correlation between smoking dose and urinary N7-MeG concentrations in our study participants. Such a result could shed light on the metabolism of cigarette smoke and the repair of adducts. The endogenous/background presence of N7-MeG adducts in DNA is well-recognized (Drabløs et al., 2004; Jenkins et al., 2005). It is possible that, at very low exposures of cigarette smoke, the biology that results in mutagenesis is driven by endogenous DNA damage. Although non-linearities in the supralinear dose–response form can occur at high doses, when exposures go beyond doses that saturate activation, detoxication, or repair, DNA adducts are expected to be linear (Swenberg et al., 2008). Exceptions to the expected linearity of DNA adducts arise when chemically induced adducts are identical to adducts that arise from endogenous sources. Furthermore, a subgroup of our smokers may partially compensate for nicotine. These smokers may adapt their smoking behavior for their individual cigarette to obtain a certain smoke (nicotine) dose. When compensating for low nicotine yields by smoking cigarettes more intensively, smokers also take in larger amounts of carcinogens from each cigarette (Benowitz et al., 2005). In addition, steady state concentrations of N7 alkylguanine adducts are achieved in 4.3 days (Walker et al., 1992). Ichiba et al. (2006) also indicated that the level of urinary N7-MeG decreased by 54% after smoking cessation for 1 week. Thus, it was also questioned whether a single measurement adequately represents the exposure of participants to methylating agents. Another explanation for the lack of correlation was the possibility of insufficient self-reporting of accumulative smoking levels because of recall bias. Consequently, exposure misclassification is likely non-differential and, if apparent, tends to underestimate methylation in DNA damage risk. Another explanation was the small number of subjects exposed to substantial levels, especially in an analysis of subgroups; hence, the power of the study was limited.

A large interindividual variation in urinary N7-MeG concentrations was identified, and this may affect the ability to detection associations. Furthermore, individual genetic variability in the enzymes that metabolize chemical carcinogens or repair DNA damage may also be involved in the mutagenic processes. Metabolic products accumulate and DNA damage persists when detoxification and DNA repair are inefficient. Lacking *GSTM1* would be expected to have higher levels of DNA adducts, although such associations are not consistently found. Previously, Loft et al. (2007) did not observe that the *GSTM1* null genotype had a greater influence on urinary N7-MeG excretion (non-null: 18.4 μmol/mmol creatinine; null: 18.3 μmol/mmol creatinine). A possible interpretation of such result was that a high load of methylating agents at a given level of smoking had no influence on the N7-MeG excretion in particular in subjects with the *GSTM1* null genotype. Lewis et al. (2004) also suggested that the *GSTM1* null genotype had a greater, although non-significant, effect (null: 9.78 N7-MeG/dG $\times 10^7$; non-null: 2.68 N7-MeG/dG $\times 10^7$) on N7-MeG in lung DNA from 38 bronchial lavage samples. In addition, Saad et al. (2006) observed an association between the *GSTM1* null genotype and lower N7-MeG adducts levels in DNA from normal bladder tissue but not tumor tissue. They also claimed that this association might occur by chance or was the result of unknown bias. However, significantly higher concentrations of urinary N7-MeG were seen in individuals with the susceptible *GSTM1* null genotype in our study. Compared to non-smokers with the *GSTM1* non-null genotype, increase but not significant urinary N7-MeG concentrations were observed in our *GSTM1* non-null smokers in the different categories of smoking dose. Our results suggest smokers that lack a functional *GSTM1* enzyme cannot detoxify tobacco smoke carcinogens well, resulting in an increase in methylation to DNA. Although it has been reported that *GSTM1* may influence NNK-induced genotoxicity (Salama et al., 1999), and *GSTM1* is known to be active against a wide variety of substrates, the exact pathways involved in N7-MeG metabolism have not been fully elucidated. In addition, there were no significant differences in mean urinary N7-MeG concentrations with the *GSTT1* and *GSTP1* genotypes, in contrast with *GSTM1* polymorphisms. This suggests that *GSTM1* may have a more significant role in tobacco smoke detoxification than *GSTT1* and *GSTP1*.

Non-smokers had detectable N7-MeG concentrations in the urine in our study; thus, environmental, occupational, or dietary exposure might be the source of these background levels (Godschalk et al., 2002; Starratt and Bond, 1988). However, no information is available on potential exposures to tobacco smoke or products, such as in non-smokers living with smokers or working with smokers or occupational exposure to smoke or automotive exhaust/diesel fumes. Although, alcohol drinking was significantly greater in smokers than in non-smokers in the present study, alcohol drinking was not associated with elevated urinary N7-MeG concentrations in either smokers or non-smokers. We did not match smokers and non-smokers on age in our study; there-

fore, the smokers were slightly younger than non-smokers. Older individuals, particularly non-smokers, had a stronger association with higher methylation in DNA. The higher methylation damage in DNA among older individuals suggests an increased susceptibility to damage with age or an accumulation of unidentified carcinogens or mutagens. Previously, Loft et al. (2007) suggested that females had higher concentrations of urinary N7-MeG, whereas Lewis et al. (2004) did not observe significantly different N7-MeG concentrations in lung DNA between males and females overall. A tendency to slightly higher concentrations of urinary N7-MeG was in observed in our female compared to male subjects. These sex differences did not reach significance. This finding was also probably due to the fact that only 29 females were included in the current study. Furthermore, we observed that individuals with a lower educational level had higher concentrations of DNA methylation. Alternative explanations for these observed associations include chance or uncontrolled confounding. It is worth speculating on the existence of unidentified risk factors (e.g., occupation) in individuals with lower educational levels.

The levels of N7-MeG in the present study were considerable lower than values published by others (Lee et al., 2008; Loft et al., 2007; Svoboda and Kasai, 2004). However, our data were also comparable to those reported by some previous studies (Farmer et al., 1993; Ichiba et al., 2006; Stillwell et al., 1991). Such discrepancy could be explained by different analytical methods and creatinine measurement. In the present study, a highly specific and sensitive isotope-dilution LC–MS/MS method coupled with an on-line solid-phase extraction system was applied. With the use of an isotope internal standard ($^{15}N_5$-N7-MeG) and on-line enrichment techniques, this method can effectively eliminate the interferences giving an accurate measurement of urinary N7-MeG, and possibly resulted in low urinary levels of N7-MeG. In addition, since urinary level of creatinine has been frequently used to adjust for variations in urinary output (Heavner et al., 2006), an accurate quantitation of urinary creatinine is also a critical factor to influence the urinary concentration of N7-MeG. This study had some limitations. For example, N7-MeG is also a product of RNA (Drabløs et al., 2004), but the relative amounts of N7-MeG that arise from RNA metabolism cannot be determined from urinary N7-MeG levels. Our results showed that cigarette smoke resulted in increased methylation to DNA. However, the concentrations of nicotine and its metabolite cotinine were not measured in our subjects. No information is provided on which types of cigarettes were smoked. We also assumed that all smokers inhaled tobacco smoke. It is also possible that the number of cigarette smoked was miscalculated from the questionnaire. Thus, misclassification of exposure might have an influence on the effect of cigarette smoking on urinary N7-MeG. Urinary concentrations of any molecular lesion rely on efficient renal excretion of the damaged products, and renal impairment can therefore affect urinary damaged DNA concentrations (Akagi et al., 2003). Urinary creatinine concentrations were used to correct for variations in urine concentrations in our study. No significant medical histories or co-morbidities were reported by our participants.

Our results suggest that smoking is associated with elevated urinary N7-MeG concentrations and that the *GSTM1* metabolic gene may modulate methylation to DNA in smokers.

Conflict of interest

There are none.

References

Akagi, S., Nagake, Y., Kasahara, J., Sarai, A., Kihara, T., Morimoto, H., Yano, A., Nakao, K., Nanba, K., Ichikawa, H., Makino, H., 2003. Significance of 8-hydroxy-2′-deoxyguanosine levels in patients with chronic renal failure. Nephrology 8, 192–195.

Avkin, S., Adar, S., Blander, G., Livneh, Z., 2002. Quantitative measurement of translesion replication in human cells: evidence for bypass of abasic sites by a replicative DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 99, 3764–3769.

Bell, D.A., Thompson, C.L., Taylor, J., Miller, C.R., Perera, F., Hsieh, L.L., Lucier, G.W., 1992. Genetic monitoring of human polymorphic cancer susceptibility genes by polymerase chain reaction: application to glutathione transferase μ. Environ. Health Perspect. 98, 113–117.

Benowitz, N.L., Jacob III, P., Bernert, J.T., Wilson, M., Wang, L., Allen, F., Dempsey, D., 2005. Carcinogen exposure during short-term switching from regular to "light" cigarettes. Cancer Epidemiol. Biomarkers Prev. 14, 1376–1383.

Bianchini, F., Montesano, R., Shuker, D.E., Cuzick, J., Wild, C.P., 1993. Quantification of 7-methyldeoxyguanosine using immunoaffinity purification and HPLC with electrochemical detection. Carcinogenesis 14, 1677–1682.

Chao, M.R., Wang, C.J., Yang, H.H., Chang, L.W., Hu, C.W., 2005. Rapid and sensitive quantification of urinary *N*7-methylguanine by isotope-dilution liquid chromatography/electrospray ionization tandem mass spectrometry with on-line solid-phase extraction. Rapid Commun. Mass Spectrom. 19, 2427–2432.

Chen, C.J., Yu, M.W., Liaw, Y.F., Wang, L.W., Chiamprasert, S., Matin, F., Hirvonen, A., Bell, D.A., Santella, R.M., 1996. Chronic hepatitis B carriers with null genotypes of glutathione *S*-transferase M1 and T1 polymorphisms who are exposed to aflatoxin are at increased risk of hepatocellular carcinoma. Am. J. Hum. Genet. 59, 128–134.

Cheng, Y.W., Chen, C.Y., Lin, P., Huang, K.H., Lin, T.S., Wu, M.H., Lee, H., 2000. DNA adduct level in lung tissue may act as a risk biomarker of lung cancer. Eur. J. Cancer 36, 1381–1388.

Chiang, S.Y., Huang, T.H., Uang, S.N., Wu, H.D., Wei, Y.C., Lin, H.Y., Swenberg, J.A., Wu, K.Y., 2005. Analysis of 7-methylguanine using isotope dilution and gas chromatography/electron-capture negative chemical ionization mass spectrometry. Rapid Commun. Mass Spectrom. 19, 1915–1920.

Drabløs, F., Feyzi, E., Aas, P.A., Vaagbø, C.B., Kavli, B., Bratlie, M.S., Peña-Diaz, J., Otterlei, M., Slupphaug, G., Krokan, H.E., 2004. Alkylation damage in DNA and RNA—repair mechanisms and medical significance. DNA Repair 3, 1389–1407.

Farmer, P.B., Bailey, E., Naylor, S., Anderson, D., Brooks, A., Cushnir, J., Lamb, J.H., Sepai, O., Tang, Y.S., 1993. Identification of endogenous electrophiles by means of mass spectrometric determination of protein and DNA adducts. Environ. Health Perspect. 99, 19–24.

Gentil, A., Cabral-Neto, J.B., Mariage-Samson, R., Margot, A., Imbach, J.L., Rayner, B., Sarasin, A., 1992. Mutagenicity of a unique apurinic/apyrimidinic site in mammalian cells. J. Mol. Biol. 227, 981–984.

Godschalk, R., Nair, J., van Schooten, F.J., Risch, A., Drings, P., Kayser, K., Dienemann, H., Bartsch, H., 2002. Comparison of multiple DNA adduct types in tumor adjacent human lung tissue: effect of cigarette smoking. Carcinogenesis 23, 2081–2086.

Haque, K., Cooper, D.P., van Delft, J.H., Lee, S.M., Povey, A.C., 1997. Accurate and sensitive quantitation of *N*7-methyldeoxyguanosine-3′-monophosphate by ^{32}P-postlabeling and storage-phosphor imaging. Chem. Res. Toxicol. 10, 660–666.

Hayes, J.D., Strange, R.C., 1995. Potential contribution of the glutathione *S*-transferase supergene family to resistance to oxidative stress. Free Radic. Res. 22, 193–207.

Hecht, S.S., 1999a. DNA adduct formation from tobacco-specific *N*-nitrosamines. Mutat. Res. 424, 127–142.

Hecht, S.S., 1999b. Tobacco smoke carcinogens and lung cancer. J. Natl. Cancer Inst. 91, 1194–1210.

Hemminki, K., 1983. Nucleic acid adducts of chemical carcinogens and mutagens. Arch. Toxicol. 52, 249–285.

Heavner, D.L., Morgan, W.T., Sears, S.B., Richardson, J.D., Byrd, G.D., Ogden, M.W., 2006. Effect of creatinine and specific gravity normalization techniques on xenobiotic biomarkers in smokers' spot and 24-h urines. J. Pharm. Biomed. Anal. 40, 928–942.

Ichiba, M., Matsumoto, A., Kondoh, T., Horita, M., Tomokuni, K., 2006. Decreasing urinary PAH metabolites and 7-methylguanine after smoking cessation. Int. Arch. Occup. Environ. Health 79, 545–549.

Jaffé, M.Z., 1886. About the precipitation caused by pikrinic acid in normal urine and about a new reaction of creatinine. Physiol. Chem. 10, 391–400 (in German).

Jenkins, G.J.S., Doak, S.H., Johnson, G.E., Quick, E., Waters, E.M., Parry, J.M., 2005. Do dose response thresholds exist for genotoxic alkylating agents? Mutagenesis 20, 389–398.

Jourenkova-Mironova, N., Wikman, H., Bouchardy, C., Voho, A., Dayer, P., Benhamou, S., Hirvonen, A., 1998. Role of glutathione *S*-transferase *GSTM1*, *GSTM3* GSTP1 and GSTT1 genotypes in modulating susceptibility to smoking-related lung cancer. Pharmacogenetics 8, 495–502.

Ketelslegers, H.B., Gottschalk, R.W., Godschalk, R.W., Knaapen, A.M., van Schooten, F.J., Vlietinck, R.F., Kleinjans, J.C., van Delft, J.H., 2006. Interindividual variations in DNA adduct levels assessed by analysis of multiple genetic polymorphisms in smokers. Cancer Epidemiol. Biomarkers Prev. 15, 624–629.

Lee, H.L., Hsueh, Y.M., Chung, C.J., Pu, Y.S., Chang, L.W., Hsieh, D.P., Liou, S.H., Lin, P., 2008. Correlation between the urine profile of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone metabolites and N^7-methylguanine in urothelial carcinoma patients. Cancer Epidemiol. Biomarkers Prev. 17, 3390–3395.

Lewis, S.J., Cherry, N.M., Niven, R.M., Barber, P.V., Povey, A.C., 2004. Associations between smoking GST genotypes and N7-methylguanine levels in DNA extracted from bronchial lavage cells. Mutat. Res. 559, 11–18.

Liao, P.C., Li, C.M., Lin, L.C., Hung, C.W., Shih, T.S., 2002. An online automatic sample cleanup system for the quantitative detection of the benzene exposure biomarker S-phenylmercapturic acid in human urine by electrospray ionization tandem mass spectrometry. J. Anal. Toxicol. 26, 205–210.

Liu, Y.J., Huang, P.L., Chang, Y.F., Chen, Y.H., Chiou, Y.H., Xu, Z.L., Wong, R.H., 2006. *GSTP1* genetic polymorphism is associated with a higher risk of DNA damage in pesticide-exposed fruit growers. Cancer Epidemiol. Biomarkers Prev. 15, 659–666.

Lodovici, M., Luceri, C., Guglielmi, F., Bacci, C., Akpan, V., Fonnesu, M.L., Boddi, V., Dolara, P., 2004. Benzo(*a*)pyrene diolepoxide (BPDE)–DNA adduct levels in leukocytes of smokers in relation to polymorphism of *CYP1A1*, *GSTM1*, *GSTP1*, *GSTT1*, and *mEH*. Cancer Epidemiol. Biomarkers Prev. 13, 1342–1348.

Loft, S., Svoboda, P., Kasai, H., Tjønneland, A., Møller, P., Sørensen, M., Overvad, K., Autrup, H., Raaschou-Nielsen, O., 2007. Prospective study of urinary excretion of 7-methylguanine and the risk of lung cancer: effect modification by *mu* class glutathione-*S*-transferases. Int. J. Cancer 121, 1579–1584.

Meer, L., Janzer, R.C., Kleihues, P., Kolar, G.F., 1986. In vivo metabolism and reaction with DNA of the cytostatic agent, 5-(3 3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC). Biochem. Pharmacol. 35, 3243–3247.

Peluso, M., Neri, M., Margarino, G., Mereu, C., Munnia, A., Ceppi, M., Buratti, M., Felletti, R., Stea, F., Quaglia, R., Puntoni, R., Taioli, E., Garte, S., Bonassi, S., 2004. Comparison of DNA adduct levels in nasal mucosa, lymphocytes and bronchial mucosa of cigarette smokers and interaction with metabolic gene polymorphisms. Carcinogenesis 25, 2459–2465.

Saad, A.A., O'Connor, P.J., Mostafa, M.H., Metwalli, N.E., Cooper, D.P., Margison, G.P., Povey, A.C., 2006. Bladder tumor contains higher N7-methylguanine levels in DNA than adjacent normal bladder epithelium. Cancer Epidemiol. Biomarkers Prev. 15, 740–743.

Salama, S.A., Abdel-Rahman, S.Z., Sierra-Torres, C.H., Hamada, F.A., Au, W.W., 1999. Role of polymorphic *GSTM1* and *GSTT1* genotypes on NNK-induced genotoxicity. Pharmacogenetics 9, 735–743.

Starratt, A.N., Bond, E.J., 1988. Methylation of DNA of maize and wheat grains during fumigation with methyl-bromide. J. Agric. Food Chem. 36, 1035–1039.

Stillwell, W.G., Glogowski, J., Xu, H.X., Wishnok, J.S., Zavala, D., Montes, G., Correa, P., Tannenbaum, S.R., 1991. Urinary excretion of nitrate, N-nitrosoproline, 3-methyladenine, and 7-methylguanine in a Colombian population at high risk for stomach cancer. Cancer Res. 51, 190–194.

Svoboda, P., Kasai, H., 2004. Simultaneous HPLC analysis of 8-hydroxydeoxyguanosine and 7-methylguanine in urine from humans and rodents. Anal. Biochem. 334, 239–250.

Swenberg, J.A., Fryar-Tita, E., Jeong, Y.C., Boysen, G., Starr, T., Walker, V.E., Albertini, R.J., 2008. Biomarkers in toxicology and risk assessment: informing critical dose–response relationships. Chem. Res. Toxicol. 21, 253–265.

Van Benthem, J., Feron, V.J., Leeman, W.R., Wilmer, J.W., Vermeulen, E., den Engelse, L., Scherer, E., 1994. Immunocytochemical identification of DNA adducts, O^6-methylguanine and 7-methylguanine, in respiratory and other tissues of rat, mouse and Syrian hamster exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis 15, 2023–2029.

van Delft, J.H., van den Ende, A.M., Keizer, H.J., Ouwerkerk, J., Baan, R.A., 1992. Determination of N7-methylguanine in DNA of white blood cells from cancer patients treated with dacarbazine. Carcinogenesis 13, 1257–1259.

Walker, V.E., MacNeela, J.P., Swenberg, J.A., Turner Jr., M.J., Fennell, T.R., 1992. Molecular dosimetry of ethylene oxide: formation and persistence of *N*-(2-hydroxyethyl)valine in hemoglobin following repeated exposures of rats and mice. Cancer Res. 52, 4320–4327.

Watson, M.A., Stewart, R.K., Smith, G.B., Massey, T.E., Bell, D.A., 1998. Human glutathione *S*-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis 19, 275–280.

Wong, R.H., Hu, C.W., Yeh, C.Y., Chao, M.R., Chen, C.C., Huang, J.H., Chang, S.H., Lee, S.I., Lee, H.S., 2008. Sulfotransferase 1A1 and glutathione *S*-transferase P1 genetic polymorphisms modulate the levels of urinary 8-hydroxy-2′-deoxyguanosine in betel quid chewers. Arch. Toxicol. 82, 313–321.