Arch Toxicol (2008) 82:313–321
DOI 10.1007/s00204-007-0248-8

TOXICOKINETICS AND METABOLISM

Sulfotransferase 1A1 and glutathione *S*-transferase P1 genetic polymorphisms modulate the levels of urinary 8-hydroxy-2′-deoxyguanosine in betel quid chewers

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Received: 11 July 2007 / Accepted: 11 September 2007 / Published online: 3 October 2007

Abstract Betel quid chewing has been associated with several human cancers. However, the role of betel quid in carcinogenesis remains uncertain. *Piper betle* contains high concentrations of safrole (an inducer of DNA oxidative damage). Safrole may be metabolized by hepatic sulfotransferase 1A1 (SULT1A1), or glutathione *S*-transferases (GSTM1, GSTT1, and GSTP1). Thus, we investigated the association of genetic polymorphisms of *SULT1A1*, *GSTM1*, *GSTT1*, and *GSTP1* with DNA oxidative damage among betel quid chewers. A biomarker for oxidative stress, urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) level, was analyzed using isotope-dilution LC–MS/MS in 64 betel quid chewers and 129 non-betel quid chewers. Data on demographics and habits (smoking, alcohol drinking, and betel quid chewing) were obtained from questionnaires. Our results revealed that urinary 8-OHdG level was higher in chewers with *SULT1A1 Arg-His* genotype than in chewers with *SULT1A1 Arg-Arg* genotype. Urinary 8-OHdG level was also higher in chewers with *GSTP1 Ile-Ile* genotype. Furthermore, the combined effect of *SULT1A1* and *GSTP1* genotypes on urinary 8-OHdG was evaluated. Non-chewers with both *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* (reference group) had the lowest mean level (3.6 ng/mg creatinine), whereas chewers with either *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* had the highest 8-OHdG mean level (6.2 ng/mg creatinine; vs. reference group, $P = 0.04$). Chewers with both of *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* (4.6 ng/mg creatinine), and non-chewers with either *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* (4.7 ng/mg creatinine) had a moderately increased 8-OHdG level. Thus, the susceptible *SULT1A1* and *GSTP1* genotypes may modulate increased DNA oxidative stress elicited by betel-quid chewing.

Keywords Betel-quid chewing · *SULT1A1* gene · *GSTP1* gene · DNA oxidative stress · Urinary 8-hydroxy-2′-deoxyguanosine

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Introduction

In Southeast Asia, especially in Taiwan and India, betel quid is a natural masticatory and stimulant composed of fresh green areca fruit, *Piper betle* (betel leaf), and slaked lime paste (Jeng et al. 2001). The prevalence of betel chewing in the Taiwanese population is greater than 10% (Ko et al. 1992). Although the chewing of betel quid is practiced in several different ways in various areas, the major components are relatively consistent. An association between betel quid with oral submucous fibrosis, oral cancer, esophageal cancer, and hepatocellular carcinoma has been demonstrated in previous studies (Chiu et al. 2002; Liu et al. 2000; Shiu et al. 2000; Tsai et al. 2001; Wu et al. 2001). However, the role of betel quid in carcinogenesis and mutagenesis remains uncertain.

In Taiwan, *Piper betle* influorescence, which contains about 15 mg/g safrole (1,2-methylenedioxy-4-allylbenzene), is frequently added to betel quid (Hwang et al. 1993). During chewing, the concentration of safrole can reach 420 mol/l. Safrole has been classified as a carcinogen in rodents (IARC 1976). Liu et al. (1999) have also indicated that safrole dose-dependently induced oxidative stress in rat liver, and that glutathione played an important protective role. The possibility of lime and area nut phenolics generated reactive oxygen species (ROS) such as hydroxyl radical (HO·) was also suggested (Nari et al. 1995). The generation of oxidative stress has been linked to chemical carcinogenesis and mutagenesis (Cerutti 1985; Kehrer 1993). Among the diverse oxidative DNA lesions, 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the most abundant base modifications and has attracted special attention because it is premutagenic, causing *G–T* transversions (Cheng et al. 1992).

In animal studies, the carcinogenicity of safrole has been attributed to the formation of safrole-DNA adducts (Borchert et al. 1973; Ioannides et al. 1981; Randerath et al. 1984; Reddy and Randerath 1990). Safrole is primarily metabolized by hepatic cytochrome P450 biotransformation to become a proximate carcinogen 1′-hydroxysafrole (Miller and Miller 1983). Furthermore, 1′-hydroxysafrole is subsequently conjugated by sulfotransferase (SULT) in liver to form its electrophilic sulfuric acid ester that reacts to form stable safrole-DNA adducts.

Metabolic polymorphisms have been implicated in the chemical exposure-related health effects. However, their exact effects on safrole-induced oxidative stress remain unclear. Sulphation catalyzed by members of the sulfotransferase enzyme family is a key metabolic pathway of many drugs, neurotransmitters, hormones, and xenobiotics (Falany 1997; Glatt 1997; Nagata and Yamazoe 2000). SULT1A1 is a major sulfotransferase enzyme in humans (Dooley et al. 1993), and Raftogianis et al. (1999) reported on a *G–A* transition that causes an *Arg* to *His* substitution at amino acid 213 of *SULT1A1*. Previous studies have shown that the *His* allele is associated with low enzyme activity compared to the wildtype *Arg* allele (Coughtrie et al. 1999; Raftogianis et al. 1999), and suggested that the high activity of the *SULT1A1 Arg* allele protects against environmental chemicals involved in the pathogenesis of cancer. Therefore, the *SULT1A1* genotype may influence susceptibility to cancer induced by environmental carcinogens such as safrole. Glutathione *S*-transferases (GSTs) protect against oxidative stress by conjugating glutathione to electrophilic species that can form protein or DNA adducts and generate ROS (Hayes and Strange 1995). Because of this important role in cellular defense against oxidative stress, genes encoding GSTs have been considered candidates for association with safrole-induced oxidative stress. The genes for *GSTM1* (Bell et al. 1992; Chen et al. 1996), *GSTT1* (Chen et al. 1996), and *GSTP1* (Watson et al. 1998) are known to be polymorphic. The *GSTM1* and *GSTT1* genotypes are also reportedly associated with an elevated risk of developing leukoplakia in Indian betel quid chewers (Nair et al. 1999). However, *SULT1A1*, *GSTM1*, *GSTT1*, and *GSTP1* metabolic genetic polymorphisms, with or without modulating effects on safrole-induced oxidative stress, have not been identified.

Thus, betel quid chewers with inherited metabolic genotypes that affect susceptibility to DNA damage may experience increased oxidative stress. We designed a population-based, cross-sectional study to investigate the association of metabolic genetic polymorphisms and urinary 8-OHdG in betel quid chewers.

Materials and methods

Study subjects and biospecimen collection

Higher prevalence of substance use (betel quid, cigarettes, and alcohol) in Taiwanese aborigines has been observed (Cheng and Chen 1995; Liu et al. 1994). Furthermore, aboriginal health status is worse than that of the general Taiwanese population. During the period from September 2003 to January 2004, a community-based, disease screening project was performed in Fuhsing, located in the northern mountain area of Taiwan. The study protocol conformed to the Declaration of Helsinki and had been approved by the relevant ethics committees of the participating institutions.

Information pertaining to personal characteristics was collected from study subjects using interviewer-administered questionnaires during the medical surveillance. Informed consent was obtained from all participants. The structured questionnaire contained questions that covered demographic characteristics and life style (including habits of cigarette smoking, alcohol drinking, and betel quid chewing). Subjects were also queried to determine amount, frequency, and duration of smoking, alcohol drinking, and betel quid chewing. Habitual alcohol drinking was defined as alcohol consumption on at least one occasion weekly and of more than 80 g of alcohol weekly as described in our previous report (Wong et al. 2002). Habitual betel quid chewing was defined as chewing one or more quids daily for at least 1 year. Additionally, in this report, obesity was evaluated using body mass index (BMI), defined as kg per m^2 (height). However, since oxidative stress has been associated with inflammatory illnesses (Crack and Taylor 2005; Dhalla et al. 2000; Nagler et al. 2003), we excluded subjects with the history of cerebrovascular or cardiovascular diseases ($n = 6$), or rheumatoid arthritis ($n = 3$), as well as

subjects who were taking antituberculosis drugs ($n = 7$), from the 209 recruited. Overall, 64 betel quid chewers and 129 non-betel quid chewers were recruited into current study. The Atayal tribe accounted for all subjects.

Urine and venous blood were collected during medical surveillance, then 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 under −70°C conditions until analysis.

Determination of urinary 8-OHdG levels

Urinary 8-OHdG level was determined as previously described by Hu et al. (2006) using liquid chromatography-tandem mass spectrometry (LC–MS/MS) coupled with automated solid-phase extraction (SPE). Briefly, the urine samples were thawed and thoroughly mixed at room temperature. After centrifugation at 5,000*g* for 5 min, 20 µl of urine was diluted tenfold with 5% (v/v) methanol containing 0.1% formic acid. To the diluted urine, we added 40 µl of $^{15}N_5$-8-OHdG solution (20 ng/ml in 5% methanol/0.1% formic acid) as internal standard followed by vigorous vortexing. Prepared urine (100 µl) was then injected into an on-line SPE LC–MS/MS. The on-line SPE system was consisted of a switching valve and an Inertsil ODS-3 column (50 × 4.6 mm, 5 µm bead size; YMC Inc., Wilmington, NC, USA). The HPLC system consisted of two series 200 micropumps, a series 200 autosampler (Perkin Elmer, Boston, MA, USA), and a Polyamine-II endcapped HPLC column (150 × 4.6 mm, 5 µm bead size, YMC) with a guard column (10 × 2 mm, YMC). An isocratic mode was used to separate the analytes. The mobile phase was 85% methanol containing 0.1% formic acid, delivered at a flow rate of 1 ml/min. The eluting of the HPLC system was introduced into a TurboionSpray source installed on a triple-quadrupole mass spectrometer (API 3000, Applied Biosystems , Foster City, CA, USA), operated in positive mode. The limit of detection (LOD) of the method was 5.7 pg/ml. Each analysis was conducted in triplicate.

Additionally, urinary levels of any oxidative lesion rely upon efficient renal excretion of the products of oxidative damage; therefore, renal impairment can affect urinary 8-OHdG levels (Akagi et al. 2003). In our study, urinary creatinine levels were used to correct for variation in urine concentration.

Polymorphisms of *SULT1A1*, *GSTM1*, *GSTT1* and *GSTP1* genes

Genomic DNA was extracted from peripheral blood of subjects. Genotyping were analyzed using polymerase chain reaction (PCR)-based methods as described below. Genotyping was also accomplished with blinding to exposure status of subjects. Rigorous quality control procedures were applied throughout the genotyping process. To avoid PCR contamination, reagents for PCR reaction were carefully aliquoted, and each aliquot was used no more than three times. For each assay, a negative control (no DNA template) was added to monitor PCR contamination. Pilot experiments were always conducted to optimize the restriction digestion conditions. After genotyping each genetic polymorphism, ~20–25% of the samples in each genotype group were randomly selected for repeated assays to validate the results.

The determination of *SULT1A1* polymorphism at amino acid 213 of exon 7 was performed as previously described (Coughtrie et al. 1999). Briefly, for the *SULT1A1* gene analysis, any restriction fragment length polymorphism (RFLP) was detected by differences in *Hae*II sites following the polymerase chain reaction (PCR) amplification. Primers used for the amplification of *SULT1A1* gene were 5′-GTT GGC TCT GCA GGG TTT CTA GGA-3′ and 5′-CCC AAA CCC CCT GCT GGC CAG CAC CC-3′. DNA (0.5 µl) was added to PCR buffer containing 200 ng of primers, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), and 0.1% of BSA in a final volume of 50 µl. Amplification was achieved by the following: denaturation (94°C for 5 min), followed by 35 cycles at 94°C for 30 s, annealing (62°C for 30 s), and extension (72°C for 30 s). The PCR products were digested with *Hae*II for 24 h at 37°C. When an *Hae*II restriction site was present, the fragment of 333 bp was digested into two lengths: 168 and 165 bp. Individuals with homozygous *His-His* had a single 333 bp fragment, whereas individuals with homozygous *Arg-Arg* had both the 168 and 165 bp fragments, and heterozygous *Arg-His* individuals had all three fragments.

GSTM1 and *GSTT1* genotypes were determined by co-amplification of two genes (Comstock et al. 1990; Pemble et al. 1994). Primers used for *GSTM1* gene were 5′-CTG CCC TAC TTG ATT GAT GGG-3′ and 5′-CTG GAT TGTAGC AGATCATGC-3′. The primers used for the *GSTT1* gene were 5′-TTC CTTACT GGT CCT CAC ATC TC-3′ and 5′-TCA CCG GAT CAT GGC CAG CA-3′. The amplification of human β-globin (110 bp) was also performed as a positive control in each reaction to confirm the presence of amplifiable DNA in the samples. The primers used for β-globin were 5′-ACA CAA CTG TGT TCA CTA GC-3′ and 5′-CAA CTT CAT CCA CGT TCA CC-3′. The amplification procedure was carried out under conditions similar to those described previously for *SULT1A1*, except denaturing was conducted at 94°C for 1 min 30 s, annealing at 59°C for 1 min, and extension at 72°C for 1 min. The reaction product was then subjected to electrophoresis in 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.

*GSTP1-Alw*26I polymorphism was also determined using a PCR–RFLP technique (Liu et al. 2006). An *Ile* to *Val* substitution in exon 5 (codon 105) was amplified to form an undigested fragment of 177 bp 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 was carried out as follows: denaturation (94°C for 30 s), annealing (61°C for 30 s), and extension (72°C for 30 s). The PCR products were digested with *Alw*26I. Homozygous *Ile-Ile* individuals had a single fragment of 177 bp, whereas homozygous *Val-Val* individuals had both 92 and 85 bp fragments, and heterozygous *Ile-Val* individuals had all three fragments.

Statistical analysis

Comparisons between betel quid chewers and non-chewers groups for age at recruitment, gender, smoking status, alcohol drinking, and BMI were made using the Student *t*-test for continuous variables and the χ^2-test for discrete variables. The χ^2-test or Fisher exact test was used to test the significance of differences in prevalence of *SULT1A1*, *GSTM1*, *GSTT1*, and *GSTP1* genotypes between betel quid chewers and non-chewers. Because the distribution of the urinary 8-OHdG level was positively skewed, we used non-parametric testing (Wilcoxon rank sum test) to test the difference in urinary 8-OHdG level for each variable. Finally, least squares means were calculated to predict the adjusted 8-OHdG levels for our study subjects with different betel quid chewing status and metabolic genotypes. All *P* values were calculated using two-tailed statistical tests.

Results

The basic characteristics of the study subjects are summarized in Table 1. Their mean age was 47.8 ± 1.1 (standard error SE) (range 17–83) years and 73.6% of the subjects were male. Betel-quid chewers were significantly younger than non-chewers (39.9 vs. 51.7; $P < 0.01$, *t*-test). The mean amount of betel-quid consumed per chewer was 9.9 ± 1.5 quids/day. The proportions of current smokers (71.9% vs. 27.9%; $P < 0.01$, χ^2-test) and alcohol drinkers (81.3% vs. 38.0%; $P < 0.01$) were significantly higher among chewers than non-chewers. A significant difference in BMI was also observed between both groups ($P = 0.04$; *t*-test).

The genotypic prevalence of *SULT1A1*, *GSTM1*, *GSTT1*, and *GSTP1* amongst the study subjects is shown in Table 2. For all subjects, the frequencies of the *Arg* and *His* alleles of *SULT1A1* were 95.6 and 4.4%, respectively, and no subjects had the *His-His* genotype. The prevalence of the *GSTM1* null-type and non-null type was 57.5 and 42.5%, respectively, and that of the *GSTT1* null-type and non-null type was 50.8 and 49.2%, respectively. In addition, the frequencies of the 105*Ile* and 105*Val* alleles of *GSTP1* were 48.7 and 51.3%, respectively. The prevalence of *SULT1A1* ($P = 0.43$, Fisher exact test), *GSTM1* ($P = 0.24$, χ^2-test), *GSTT1* ($P = 0.29$), and *GSTP1* ($P = 0.75$) genotypes did not differ significantly between chewers and non-chewers.

The creatinine-adjusted mean urinary 8-OHdG level for the study subjects was 4.4 ± 0.3 ng/mg (SE) creatinine (Table 3). Mean urinary 8-OHdG levels for the betel-quid

Table 2 Genotype prevalence of *SULT1A1*, *GSTM1*, *GSTT1*, and *GSTP1* in betel-quid chewers and non-chewers

Variables	Non-chewers ($n = 129$)	Betel-quid chewers ($n = 64$)	Total ($n = 193$)
SULT1A1			
Arg-Arg	116 (89.9%)	60 (93.8%)	176 (91.2%)
Arg-His	13 (10.1%)	4 (6.2%)	17 (8.8%)
His-His	0 (0.0%)	0 (0.0%)	0 (0.0%)
GSTM1			
Null	78 (60.5%)	33 (51.6%)	111 (57.5%)
Non-null	51 (39.5%)	31 (48.4%)	82 (42.5%)
GSTT1			
Null	62 (48.1%)	36 (56.3%)	98 (50.8%)
Non-null	67 (56.3%)	28 (43.7%)	95 (49.2%)
GSTP1			
Ile-Ile	39 (30.2%)	16 (25.0%)	55 (28.5%)
Ile-Val	51 (39.6%)	27 (42.2%)	78 (40.4%)
Val-Val	39 (30.2%)	21 (33.8%)	60 (31.1%)

Number of subjects (%)

Table 1 Basic characteristics of betel-quid chewers and non-betel-quid chewers

Variables	Non-chewers ($n = 129$)	Betel-quid chewers ($n = 64$)	Total ($n = 193$)
Age	51.7 ± 1.3	39.9 ± 1.4**	47.8 ± 1.1
Gender: male	90 (69.8%)	52 (81.3%)	142 (73.6%)
Current smokers	36 (27.9%)	46 (71.9%)**	82 (42.5%)
Cigarettes/day	6.0 ± 0.9	15.8 ± 1.6**	9.2 ± 0.9
Alcohol drinkers	49 (38.0%)	52 (81.3%)**	101 (52.3%)
Alcohol consumption (g/wk)	81.2 ± 16.9	285.3 ± 108.4	148.9 ± 38.1
Body mass index (kg/m^2)	24.6 ± 0.3	25.8 ± 0.5*	24.9 ± 0.2

Mean ± standard error
Number of subjects (%)
* $0.01 < P < 0.05$
** $P < 0.01$

Table 3 Urinary 8-OHdG level (ng/mg creatinine) stratified by betel-quid chewing status and various factors

Variables	Non-chewers		Betel-quid chewers		Total	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
All	129	4.3 ± 0.5	64	4.6 ± 0.4	193	4.4 ± 0.3
Age						
≥48 years	80	4.7 ± 0.7	12	4.9 ± 0.7	92	4.7 ± 0.6
<48 years	49	3.5 ± 0.4	52	4.5 ± 0.5	101	4.0 ± 0.3
Gender						
Males	90	3.6 ± 0.2	52	4.5 ± 0.4	142	3.9 ± 0.2
Females	39	5.9 ± 1.4	12	4.7 ± 1.3	51	5.6 ± 1.1
Current smoking						
Yes	36	3.5 ± 0.4	46	5.2 ± 0.5**	82	4.5 ± 0.4
No	93	4.5 ± 0.6	18	3.1 ± 0.5	111	4.3 ± 0.5
Alcohol drinking						
Yes	49	3.9 ± 0.4	52	4.6 ± 0.5	101	4.3 ± 0.3
No	80	4.5 ± 0.7	12	4.3 ± 0.9	92	4.5 ± 0.6
BMI						
≥ 25 kg/m^2	54	5.1 ± 1.1	36	4.5 ± 0.6	90	4.9 ± 0.7
< 25 kg/m^2	75	3.6 ± 0.3	28	4.6 ± 0.6	103	3.9 ± 0.3
SULT1A1 genotype						
Arg-His	13	3.1 ± 0.8	4	6.6 ± 3.2	17	3.9 ± 1.0
Arg-Arg	116	4.4 ± 0.5	60	4.4 ± 0.4	176	4.4 ± 0.4
GSTM1						
Null	78	4.4 ± 0.8	33	4.1 ± 0.5	111	4.3 ± 0.5
Non-null	51	4.1 ± 0.4	31	5.0 ± 0.6	82	4.4 ± 0.3
GSTT1						
Null	62	3.7 ± 0.3	36	4.9 ± 0.6	98	4.2 ± 0.3
Non-null	67	4.7 ± 0.9	28	4.2 ± 0.5	95	4.6 ± 0.6
GSTP1 genotype						
Ile-Ile	39	5.5 ± 1.4	16	5.3 ± 0.7	55	5.4 ± 1.0
Val-Val/Ile-Val	90	3.7 ± 0.3	48	4.3 ± 0.5	138	3.9 ± 0.3

Comparison between different age, gender, smoking status, alcohol drinking, BMI, and genotype groups conducted using the Wilcoxon rank sum test

** $P < 0.01$

chewers and non-chewers were 4.6 (range 0.5–16.1) and 4.3 ng/mg (range 0.1–57.5) creatinine. Among chewers, urinary 8-OHdG level was obviously higher in those with cigarette smoking habits than in those without cigarette smoking habits (5.2 vs. 3.1 ng/mg creatinine; $P < 0.01$). In addition, the urinary 8-OHdG level was higher in chewers with *SULT1A1 Arg-His* genotype than in chewers with *SULT1A1 Arg-Arg* genotype (6.6 vs. 4.4 ng/mg creatinine), but this difference did not reach statistical significance ($P = 0.14$). In addition, *GSTP1 Ile-Val* and *Val-Val* genotypes were combined because it has been shown that individuals with at least one *GSTP1 Val* allele have a lower enzyme activity than those with the *GSTP1 Ile* allele (Zimniak et al. 1994). The urinary 8-OHdG level was also higher in chewers with *GSTP1 Ile-Ile* genotype (vs. *Ile-Val/Val-Val*, $P = 0.09$). However, age, gender, alcohol drinking status, BMI, *GSTM1* genotype, and *GSTT1* genotype were not associated with elevated urinary 8-OHdG.

Furthermore, a least-squares mean analysis was performed to assess the urinary 8-OHdG level of chewers who possessed the metabolic *SULT1A1*, *GSTM1*, *GSTT1*, and *GSTP1* genotypes after adjusting for age, gender, smoking, alcohol drinking, and BMI (Table 4). When non-chewers with *SULT1A1 Arg-Arg* genotype were selected as reference (4.2 ng/mg creatinine), chewers with *SULT1A1 Arg-Arg* genotype had an increased level of urinary 8-OHdG (4.9 ng/mg creatinine). Chewers with *SULT1A1 Arg-His* genotype had the highest level of urinary 8-OHdG (7.1 ng/mg creatinine; 95% CI = 2.2–11.9). However, the differences in different combinations did not reach statistical significance. Similarly, non-chewers with *GSTP1 Val-Val/Ile-Val* genotype had the lowest level of urinary 8-OHdG (3.5 ng/mg creatinine). Groups ordered according to urinary 8-OHdG level (from highest to lowest) were: chewers with *GSTP1 Ile-Ile* genotype (6.0 ng/mg creatinine; $P = 0.08$), non-chewers with *GSTP1 Ile-Ile* genotype

Table 4 Adjusted urinary 8-OHdG levels (ng/mg creatinine) by betel-quid chewing status and different metabolic genotypes

Variables	Non-chewers			Betel-quid chewers		
	N	Adjusted mean (95% CI)	*P*-Value	*n*	Adjusted mean (95% CI)	*P*-Value
SULT1A1						
Arg-His	13	2.8 (0.1–5.4)	0.32	4	7.1 (2.2–11.9)	0.25
Arg-Arg	136	4.2 (3.2–5.1)	Ref	60	4.9 (3.5–6.3)	0.40
GSTM1						
Null	78	3.5 (2.2–4.7)	0.23	33	5.4 (3.7–7.1)	0.44
Non-null	51	4.5 (3.3–5.7)	Ref	31	4.7 (2.7–6.6)	0.90
GSTT1						
Null	62	4.2 (3.0–5.3)	0.72	36	4.7 (2.9 –6.5)	0.47
Non-null	67	3.8 (2.5–5.2)	Ref	28	5.4 (3.6–7.2)	0.19
GSTP1						
Ile-Ile	39	5.2 (3.6–6.7)	0.07	16	6.0 (3.5–8.5)	0.08
Val-Val/Ile-Val	90	3.5 (2.5–4.5)	Ref	48	4.8 (3.3–6.2)	0.19

Adjusted the effects of age, gender, smoking, alcohol drinking, and BMI

(5.2 ng/mg creatinine; $P = 0.07$), non-chewers with *GSTP1 Val-Val/Ile-Val* genotype (4.8 ng/mg creatinine, $P = 0.19$), and non-chewers with *GSTP1 Val-Val/Ile-Val* genotype (3.5 ng/mg creatinine; reference group). There were no obvious differences in urinary 8-OHdG level between chewers and non-chewers with different *GSTM1* and *GSTT1* genotypes.

Subsequently, the combined effect of *SULT1A1* and *GSTP1* genotypes on urinary 8-OHdG was also evaluated (Table 5). Non-chewers with both *SULT1A1 Arg-Arg* genotype and *GSTP1 Val-Val/Ile-Val* genotypes (reference group) had the lowest mean level (3.6 ng/mg creatinine; $n = 80$), whereas chewers with either *SULT1A1 Arg-His* genotype or *GSTP1 Ile-Ile* genotype had the highest 8-OHdG (6.2 ng/mg creatinine; $n = 19$; vs. reference group, $P = 0.04$). Chewers with both of *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* (4.6 ng/mg creatinine; $n = 45$), and non-chewers with either *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* (4.7 ng/mg creatinine; $n = 49$) had a moderately increased 8-OHdG mean level. Furthermore, the difference in urinary 8-OHdG levels between the reference group and chewers with either *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* (2.6 ng/mg creatinine) was greater than the combined differences in urinary 8-OHdG levels between the reference group and chewers with both of *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* (1.0 ng/mg creatinine), and between the reference group and non-chewers with either *SULT1A1 Arg-His* genotype or *GSTP1 Ile-Ile* genotype (1.1 ng/mg creatinine).

Discussion

Betel-quid chewing is a popular oral habit and it has been associated with several human cancers in epidemiological studies (Liu et al. 2000; Shiu et al. 2000; Tsai et al. 2001; Wu et al. 2001). However, the role of betel-quid in carcinogenesis and mutagenesis remains unclear. In our study, urinary 8-OHdG levels were significantly higher in betel-quid chewers possessing *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* genotypes than in non-chewers with *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* genotypes.

Piper betel contains high concentrations of safrole (Hwang et al. 1993), which may induce oxidative stress (Liu et al. 1999). Previous animal studies revealed that safrole is primarily metabolized by the hepatic cytochrome P450 system to become active intermediate 1′-hydroxysafrole (Borchert et al. 1973; Ioannides et al. 1981; Randerath

Table 5 Adjusted urinary 8-OHdG levels (ng/mg creatinine) by betel-quid chewing status with combined *SULT1A1* and *GSTP1* genotypes

Combined genotypes	Non-chewers			Betel-quid chewers		
	n	Adjusted mean (95% CI)	*P*-value	*n*	Adjusted mean (95% CI)	*P*-value
SULT1A1 Arg-His or *GSTP1 Ile-Ile*	49	4.7 (3.3–6.1)	0.19	19	6.2 (3.9–8.5)	0.04
SULT1A1 Arg-Arg and *GSTP1 Val-Val/Ile-Val*	80	3.6 (2.5–4.7)	Ref	45	4.6 (3.1–6.1)	0.30

Adjusted the effect of age, gender, smoking, alcohol drinking, and BMI

Included individuals with both of *SULT1A1 Arg-His* and *GSTP1 Ile-Ile* genotypes, those with both of *SULT1A1 Arg-His* and *GSTP1 Val-Val/Ile-Val* genotypes, and those with both of *SULT1A1 Arg/Arg* and *GSTP1 Ile/Ile* genotypes

et al. 1984; Reddy and Randerath 1990). This proximate carcinogen is subsequently conjugated by sulfotransferase to form electrophilic sulfuric acid ester. SULT1A1 is a major sulfotransferase enzyme in humans (Dooley et al., 1993), thus, the *SULT1A1* genotype may influence susceptibility to carcinogenicity or mutagenicity following betel-quid chewing. Previous studies have also shown that the *SULT1A1 His* allele is associated with low enzyme activity compared to the *Arg* allele (Coughtrie et al. 1999; Raftogianis et al. 1997). Furthermore, a significantly reduced risk of cancer was observed to be associated with homozygosity for *SULT1A1 Arg* allele (Bamber et al. 2001; Wu et al. 2003). These results suggest that the high activity of the *SULT1A1 Arg* allele protects against environmental chemicals involved in the pathogenesis of cancer. From our data, chewers possessing *SULT1A1 Arg/His* had a lower metabolic activity than those with *SULT1A1 Arg/Arg* genotype, and thus may have elevated levels of active intermediates and oxidative stress. However, regarding the association of *SULT1A1* genetic polymorphism with DNA oxidative stress in this study, the possibility of a type II error as a result of insufficient statistical power cannot be ruled out; thus, further study with a larger sample size is required.

When excessive quantities of ROS are produced, crucial cellular macromolecules (such as DNA) may be attacked and thereby cell function may become impaired. Glutathione *S*-transferases (GSTs) protect against oxidative stress by conjugating glutathione to electrophilic species that can form protein or DNA adducts and generate ROS (Hayes and Strange 1995). Because of this important role in cellular defense against oxidative stress, genes encoding GSTs have been considered as candidates for association with safrole-induced oxidative stress. Interestingly, individuals with the *GSTP1 Ile-Ile* genotype had increased DNA oxidative stress in our study. The functional effect of the *GSTP1 Ile*105-*Val*105 substitution may be substrate dependent. Compared with Val-containing enzymes, Ile-containing GSTP1 was associated with a threefold increase in specific activity towards 1-chloro-3,4-dinitrobenzene, but a sevenfold reduction in activity towards polycyclic aromatic hydrocarbons (Hu et al. 1997; Watson et al. 1998). Thus, GSTP1 Ile-containing enzymes are less efficient than Val-containing enzymes at detoxifying the products of oxidative stress elicited by betel-quid chewing. Functional studies will be required to test these hypotheses. In addition to *GSTP1*, no significant difference in urinary 8-OHdG level was found between subjects with *GSTM1* and *GSTT1* genotype in our study. Such differences could result from differences in the type and/or level of expression of individual GST isoforms, and/or specific differences in activity of enzymes necessary to form the GST substrate.

Interestingly, DNA oxidative stress in our study was more likely in chewers with either both *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* than in non-chewers with both *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* (Table 5). Chewers with both of *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* had a moderately increased 8-OHdG level. Since *SULT1A1* and *GSTP1* are involved in the biotransformation of betel quid, and act as detoxifying enzymes for the reactive metabolites, this finding indicates that while the combination of *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* seem to protect chewers from DNA oxidative stress, the enzymes separately may provide only minor protection. In addition, we observed a synergistic effect of *SULT1A1* and *GSTP1* genotypes on DNA oxidative stress in chewers in the current study. This implies that betel quid causes other types of cellular damage, or affects other defenses such as repair enzymes against carcinogenesis. Additional study including more subjects may shed light on these questions.

Aging is reportedly associated with increased urinary 8-OHdG levels (Adelman et al. 1988; Cathcart et al. 1984). As the balance between pro-oxidant and antioxidant processes is shifted in favor of the former during aging, more 8-OHdG is generated from DNA oxidation and ring opening followed by rearrangements (Cadet et al. 2003). In our analysis, older individuals also had higher urinary 8-OHdG than did younger individuals. Since cigarette smoke (Loft et al. 1992) and alcohol (Bailey and Cunningham 1998) contain ROS, it would be expected that oxidative stress is increased in subjects with these habits. However, we did not find statistical association between alcohol drinking and increased urinary 8-OHdG. Loft et al. (1992) reported that overweight persons had lower metabolic rates than did lean persons. Our study did not reveal a significant association between BMI and 8-OHdG, probably because most of our subjects were not obese (mean BMI, 24.9).

There were some limitations to this study. The amount of the modified base in cellular DNA excreted into urine should represent the average rate of DNA damage in the whole body (Cooke et al. 2000). Moreover, it is possible that the levels of oxidative DNA damage are reflective of different active diseases. Since urinary levels of any oxidative lesion rely upon efficient renal excretion of the products of oxidative damage, renal impairment can therefore affect urinary 8-OHdG level (Akagi et al. 2003). In our study, urinary creatinine levels were used to correct for variation in urine concentration. In addition, no medical histories were reported by our participants. Furthermore, in Taiwan, higher educational level and socioeconomic status is associated with lower likelihood of betel quid chewing (Ko et al. 1992). We tried to minimize possible selection bias by recruiting control subjects from the same geographic area and having the same ethnicity and similar socioeconomic status as the study subjects. Though decreased levels of cellular oxidative damage have been found in those subjects with increased intake of antioxi-

dants-rich vegetables and fruits (Thompson et al. 1999), no data were collected or available on fruit and vegetable intake. In addition, we deemed the available historical exposure data too sparse and lacking in detail for a quantitative estimation for amount and duration of betel quid chewed. Data pertaining to individual exposure was obtained without the knowledge of health outcome. Consequently, exposure misclassification is assumed to be non-differential and, if apparent, directed toward an underestimation of the risk for oxidative DNA damage. Lastly, our study was limited by the relative small numbers of subjects, especially in the analysis of subgroups. In the beginning of our study, research assistants abstracted name, personal identification number, gender, date of birth from the records of local housing offices. A total of 700 residents in our study area were invited to participate. However, the response rate was low. To improve the response rate, we used a variety of strategies, including sending letters when phones were disconnected, sending research staff to the last known address, and using contacts (friends and neighbors) to gets updated information on the participants or to pass a message along. The reasons given from subjects who rejected to or could not participate in the study were that they were not busy for the interview, were out of town, could not be located, and were unwilling to provide the biospecimens.

In our study, increased generation of 8-OHdG was found in betel-quid chewers with *SULT1A1* and *GSTP1* genotypes that affect susceptibility to DNA damage. However, the role of other metabolic genes on oxidative DNA damage also requires further study. In the future, longitudinal rather than cross-sectional studies should be conducted to ascertain the possible association between betel-quid chewing and oxidative DNA lesions. A longitudinal study that includes a sufficient number of participants has the potential to show dose–effect relationships.

Acknowledgments The authors thank local nurses in the Health Center of Fuhsing for helpful assistance. This study was supported by CSMU 95-OM-B-002, Taiwan.

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