Association between *GST* Genetic Polymorphism and Dose-Related Production of Urinary Benzene Metabolite Markers, *trans, trans*-Muconic Acid and *S*-Phenylmercapturic Acid

Lung-Cheng Lin,[1] Wan-Jou Chen,[1] Yin-Mei Chiung,[3,4] Tung-Sheng Shih,[3] and Pao-Chi Liao[1,2]

[1]Department of Environmental and Occupational Health, College of Medicine, and [2]Sustainable Environment Research Center, National Cheng Kung University, Tainan, Taiwan; [3]Institute of Occupational Safety and Health, Shijr City, Taiwan; and [4]Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan

Abstract

The urinary benzene metabolites, *trans, trans*-muconic acid (ttMA) and *S*-phenylmercapturic acid (SPMA), are widely used as benzene exposure biomarkers. The influence of the glutathione *S*-transferase (*GST*) genetic polymorphism on the excretion levels of urinary ttMA and/or SPMA has been investigated. The association between dose-related production of urinary benzene metabolites and benzene exposure level was also reported. However, the association between the dose-related productions of urinary benzene metabolites and *GST* genetic polymorphism was not described in the literature. The purpose of this study was to investigate the association between the *GST* genetic polymorphism and dose-related production of the two widely used biomarkers, urinary ttMA and SPMA. Seventy male workers in a chemical factory were measured for their benzene exposure levels and provided blood and urine specimens at the end of work-shift. The atmospheric benzene exposure levels of these workers were determined by passive samplers with gas chromatograph mass spectrometer. The urinary ttMA and SPMA levels were quantitated by an online dual-loop cleanup device with an electrospray ionization tandem mass spectrometer. The analyses of *GST* genotypes, including M_1, T_1, and P_1, were done using PCR. Mean ($\pm$ SD) of benzene exposure levels in participants was 7.2 $\pm$ 15 ppm. The ttMA and SPMA levels in the high benzene exposure group ($\geqq$1 ppm) were higher than those in the low benzene exposure group (<1 ppm; $P < 0.001$). Among the *GST* genotypes investigated in this study, the results showed that only the *GSTT1* genotype was related to the level and dose-related production of SPMA. Using SPMA for evaluating benzene exposure, the results suggest that the *GSTT1* genetic polymorphism, especially in a comparison study between two populations with different *GSTT1* genotype frequencies, should be considered. Additionally, the biological exposure index value of SPMA should be set based on the levels of subjects with *GSTT1*-deficient genotypes for protection of all subjects. (Cancer Epidemiol Biomarkers Prev 2008;17(6):1460–9)

Introduction

Benzene has been classified as a human carcinogen with hematoxicity by IARC (1). Exposure to benzene is not only associated with cancer, such as leukemia, lung cancer, and lymphoma, but also associated with aplastic anemia. As shown in Fig. 1, the metabolism of benzene has been comprehensively investigated (2, 3). Among benzene metabolites, urinary *trans, trans*-muconic acid (ttMA) and *S*-phenylmercapturic acid (SPMA) were reported to provide good specificity for benzene exposure and often used to evaluate the internal dose in exposed workers (4). The American Conference of Governmental Industrial Hygienists (ACGIH) has set biological exposure indices (BEI) values for ttMA and SPMA as 500 and 25 $\mu g\ g^{-1}$ creatinine, respectively, for biological monitoring (5). Both of these BEI values were based on the time-weighted average of 0.5-ppm benzene exposure.

The glutathione *S*-transferase (*GST*) supergene family consists of several gene subfamilies including *GSTM1*, *GSTT1*, and *GSTP1*. The *GSTM1* and *GSTT1* genes both display deletion polymorphism. Homozygous deletions of *GSTM1* gene (*GSTM1*-deficient genotype) and *GSTT1* gene (*GSTT1*-deficient genotype) result in the absence of enzyme activity (6). In *GSTP1* genotype, a single nucleotide substitution (A to G) at nucleotide 313, which causes the change of isoleucine to valine, decreases the *GSTP1* enzyme activity (7).

The genetic polymorphism of *GST* may be related to health effects of benzene exposure and the levels of urinary benzene metabolites. In a benzene-exposed population, the *GSTT1*-deficient genotype was found to be a good predictor of diepoxybutane-induced sister chromatid exchange and a significant benzene-*GSTT1* interaction was observed (8). In a population exposed to hydroquinone, a metabolite of benzene, the individuals

Received 2/19/08; accepted 3/17/08.

Grant support: Institute of Occupational Safety and Health, National Science Council grants NSC93-2113-M-006-017 and NSC94-2113-M006-06, and NCKU Project of Promoting Academic Excellence and Developing World Class Research Centers from Ministry of Education of Taiwan.

Requests for reprints: Pao-Chi Liao, Department of Environmental and Occupational Health, College of Medicine, National Cheng Kung University, 138 Sheng-Li Road, Tainan 704, Taiwan. Phone: 886-6-2353535, ext. 5566; Fax: 886-6-2743748. E-mail: liaopc@mail.ncku.edu.tw

doi:10.1158/1055-9965.EPI-08-0160

with *GSTM1*-deficient genotype had a significantly higher frequency of micronuclei, an index of genotoxicity (9). A line of evidence indicated that *GSTT1*- and *GSTM1*-deficient genotypes tended to be more susceptible to benzene toxicity (10-12). In addition, it has been reported that the *GST* genotype polymorphism influenced the levels of urinary ttMA and/or SPMA (13-17). Furthermore, in a lower benzene–exposed population (0.015 ppm), higher levels of urinary ttMA were shown in subjects with *GSTM1*-deficient genotype than those in subjects with *GSTM1*-positive genotype (14). These studies suggest that the *GST* genotype polymorphism can influence the excretion of urinary ttMA and SPMA (13-17).

The association between dose-related production of urinary benzene metabolites and benzene exposure level was described in a recent report published in 2006 (18). The dose-related production of metabolite represents the amount of metabolite generated from the per unit benzene exposure dose. The report showed that these urinary benzene metabolites, including phenol, hydroquinone, catechol, and ttMA, were related to saturable metabolic pathways, except SPMA. However, the association between the dose-related production of urinary benzene metabolites and *GST* genetic polymorphism was not discussed in the report. Therefore, the objective of this study was to investigate the association between the *GST* genetic polymorphism and dose-related production of the two widely used biomarkers, urinary ttMA and SPMA.

Materials and Methods

Subjects. The study was conducted in a chemical synthesis factory in Taiwan, and the study population consisted of 105 male subjects. Each subject completed a self-report questionnaire and provided blood and urine samples. Information about demographic data, including age, diet, smoking habit, work situation, and equipment-using situation, was obtained. The blood samples were treated with EDTA and analyzed for *GST* genotypes. The urine samples were analyzed for levels of ttMA, SPMA, and creatinine. Twenty-nine subjects were excluded under the criteria that their urinary creatinine levels were out of the range of 0.30 to 3.0 g L^{-1}. Because smoking behavior is considered a confounding factor, and only six smokers are in the population, the six smokers were excluded from the population. Finally, 70 subjects were involved in this study. Informed consents were given by participating subjects, and the study protocol was approved by the medical ethic committee of the National Cheng Kung University Hospital, Tainan, Taiwan.

Air Benzene Sampling and Analysis. The time-weighted average concentration of benzene was measured by a diffusive sampling method. The diffusive samplers (vapor monitor 3500) were obtained from 3M. Each subject wore a diffusive sampler on the lapel during their work-shift. At the end of the work-shift, the samplers were collected and sealed with the parafilm tie to prevent loss of the analyte. Before instrumental analysis, 2.0 μg of $^{13}C_1$-labeled toluene were added into the diffusive sampler as internal standard. The benzene was extracted from the diffusive sampler with 2.0-mL carbon disulfide. The 1.0-μL extracted carbon disulfide was injected into a gas chromatograph with electron impact ion trap mass spectrometer (Finnigan Corporation). The *m/z* of benzene and $^{13}C_1$-labeled toluene were 78 and 92, respectively. The calibration curve was constructed by the different concentration of benzene plots with same amount of $^{13}C_1$-labeled toluene.

The apparent recovery was done per 10 sample runs with spiked samples. The precision of samples was also determined by replicate analysis. The ranges of apparent recovery and precision were 101% to 105% and 0.55% to 10%, respectively. Desorption efficiencies of the diffusive sampler, estimated at three concentrations, ranged from 97% to 103%.

Urine Sampling and Analysis. These urine samples were collected at the end of work-shift. The urine samples were collected in 15-mL polypropylene tube and kept at 4°C after collection at the factory. All urine samples were stored at −20°C before analysis. The collection of these urine samples was according to the ACGIH commendation (5).

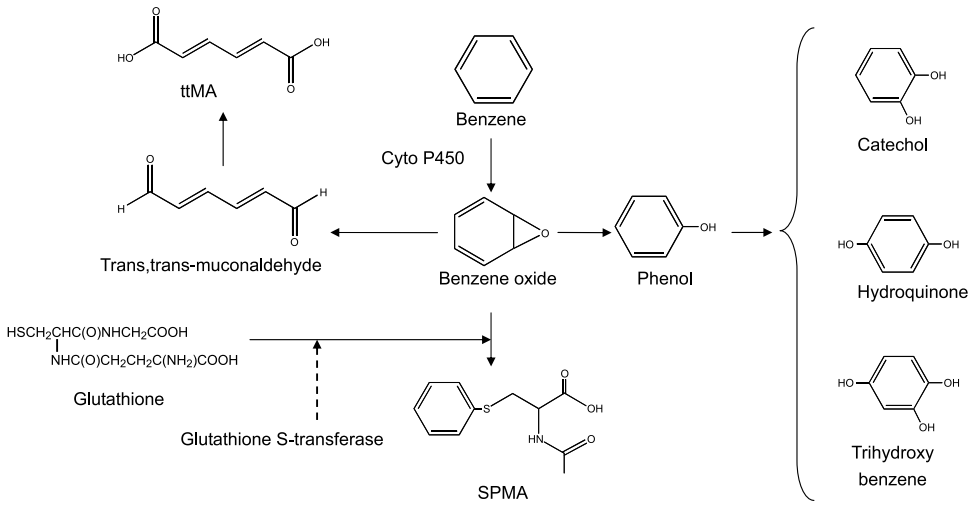

Figure 1. Benzene metabolism and major function of *GST*.

Table 1. Characteristics of the study population

Characteristics	
Age (y)	
Mean ± SD	45 ± 7.8
Range	33-57
Work seniority (mo)	
Mean ± SD	200 ± 74
Range	3-300
GSTM1, n (%)	
Positive	38 (54)
Deficient	32 (46)
GSTT1, n (%)	
Positive	33 (47)
Deficient	37 (53)
GSTP1, n* (%)	
ile/ile	42 (60)
ile/val	25 (36)
val/val	2 (2.8)

*One sample loss.

Urine sample preparation and analysis procedures were done by an online cleanup device with electrospray ionization tandem mass spectrometer for quantitation of urinary ttMA and SPMA (19).

The samples added to BEI concentration of these two analytes were analyzed for examination of apparent recoveries. The recovery values of these two analytes were in the range of 100 ± 15%. The precisions of these two urinary analytes were tested by triplicate repeat analyses. The relative SDs of the two analytes were both <10%. The ttMA and SPMA levels of each subject were adjusted by their urinary creatinine levels.

Genotype Analysis. Before DNA storage, the DNA was isolated from each blood sample using XNAB REDExtract-N-Amp Blood PCR Kit (a DNA isolation kit, provided by Sigma Chemical). The *GSTM1* and *GSTT1* genotype polymorphisms were determined according to Dirksen et al. (20). The *GSTP1* genotype polymorphism was determined according to Harries et al. (21). These genotypes were detected by PCR, and then the samples were separated electrophoretically on 2.5% agarose gels and visualized by UV-induced luminescence. For *GSTP1* genotype analysis, after the PCR process, the PCR product was digested with *Bsm*AI, an isoschizomer of *Alw*26I (New England BioLabs), and then electrophoretical separation was done. According to the *GSTP1* genotype polymorphism associated with the amino acid replacement, the *GSTP1* genotypes were shown by ile/ile, ile/val, and val/val, the replacements of amino acid sequence. Additionally, albumin was used in PCR and electrophoretical separation processes as a positive control.

Statistical Methods. PC software, SPSS version 12, was used for statistical tests and regression analyses. Because the levels of these two urinary metabolites had geometric distributions, the data were log transformed for further statistical comparison and correlation analyses. Independent-sample *t* test was used to analyze the two urinary metabolite levels of subjects with *GSTM1*- and/or *GSTT1*-positive genotypes compared with deficient genotypes. In *GSTP1* group, the Kruskal-Wallis test was used to analyze the metabolite levels among the three *GSTP1* subgroups, including ile/ile, ile/val, and val/val, because of the small sample size in val/val ($n = 2$). For comparing the dose-related production of urinary metabolite in the two *GSTT1* genotypes, a statistical method that was reported by Tsutakawa and Hewett (22) was used. $P < 0.05$ was considered statistically significant.

Results

Characteristics of the Study Population. The characteristics of the study population, including age and work seniority, were obtained with a self-report questionnaire and summarized in Table 1. The average age of the population was 45 ± 7.8 years (mean ± SD). The mean work seniority of the population was 200 ± 74 months.

The frequencies of the *GSTM1, GSTT1*, and *GSTP1* alleles were also given in Table 1. Both of the *GSTM1* and *GSTT1* allele frequencies that were close to 50% in this population were similar to those reported previously in Chinese and Taiwanese populations (23, 24). The *GSTP1 ile/ile, ile/val*, and *val/val* allele frequencies were 60%, 36%, and 2.8%, respectively. The occurrence of *GSTP1* allele frequency was also similar to the occurrence reported in a Chinese control group (24).

Levels of Benzene Exposure and Urinary Metabolites. The levels of atmospheric benzene exposure and its urinary metabolites were shown in Table 2. The benzene time-weighted average concentration of the population was 7.2 ± 15 (mean ± SD, ppm) and ranged from 5.2 ppb to 70 ppm with a median of 0.62 ppm. These subjects were divided into two subgroups based on whether the benzene exposure levels were <1 or ≧1 ppm for three following reasons: First, these subjects were divided for approximately equal subject sizes. Second, for comparing the levels of urinary benzene metabolites to the metabolite levels in the previous report at similar exposure level, the cutoff point of benzene exposure level was set at 1 ppm (29). Third, ttMA could be reliable in detecting benzene exposure level down to 1 ppm (4, 25). There were 33 subjects with high benzene exposure (≧1 ppm) and 37 subjects with low exposure (<1 ppm). The mean benzene exposure levels of the high and low exposure groups were 15 ± 19 and

Table 2. Levels of benzene exposure and its urinary metabolites, ttMA and SPMA

Variables	Mean ± SD (range)
Entire study population ($n = 70$)	
Atmospheric benzene (ppm)	7.2 ± 15 (0.0052-70)
ttMA (μg/g creatinine)	5,300 ± 12,000 (48-67,000)
SPMA (μg/g creatinine)	16 ± 32 (0.78-190)
High benzene exposure (≧1 ppm; $n = 33$)	
Atmospheric benzene (ppm)	15 ± 19 (1.0-70)
ttMA (μg/g creatinine)	10,000 ± 16,000* (1,100-67,000)
SPMA (μg/g creatinine)	29 ± 43† (3.3-190)
Low benzene exposure (<1 ppm; $n = 37$)	
Atmospheric benzene (ppm)	0.20 ± 0.22 (0.0052-0.90)
ttMA (μg/g creatinine)	590 ± 480* (48-1,700)
SPMA (μg/g creatinine)	4.1 ± 5.2† (0.78-24)

NOTE: Statistical comparison: independent-sample *t* test. The levels of urinary ttMA and SPMA were log transformed.
*ttMA level between high and low exposure groups, $P < 0.001$.
†SPMA level between high and low exposure groups, $P < 0.001$.

0.20 ± 0.22 ppm, respectively. Additionally, the benzene exposure level shows significant difference between these two groups.

In all of urinary samples, ttMA and SPMA levels were determined above the detection limits (19). Among the study subjects, ttMA levels of 49 subjects were higher than the corresponding BEI value (500 μg g^{-1} creatinine), but only nine subjects' SPMA levels were higher than the BEI value (25 μg g^{-1} creatinine). In the high exposure group (≧1 ppm), the ttMA levels of all subjects were higher than the BEI value. However, there were only nine subjects in the high exposure group whose SPMA levels were higher than the corresponding BEI value. By comparing the levels of the two metabolites between the high (≧1 ppm) and low (<1 ppm) exposure groups, both ttMA and SPMA levels were significantly higher in the high exposure group than in the low exposure group (Table 2; $P < 0.001$).

As shown in Fig. 2, significant correlations between time-weighted average benzene concentration and urinary ttMA levels ($r = 0.731$, $P < 0.05$), and between benzene concentration and SPMA ($r = 0.762$, $P < 0.05$), in the entire study group were observed. The ttMA and SPMA levels were geometrically distributed with urinary creatinine adjustment. Therefore, to conduct further correlation analysis between these two metabolites, the ttMA and SPMA levels were geometrically transformed. The levels of these two metabolites after geometric transformation showed a high correlation (Fig. 3; $r = 0.823$, $P < 0.05$).

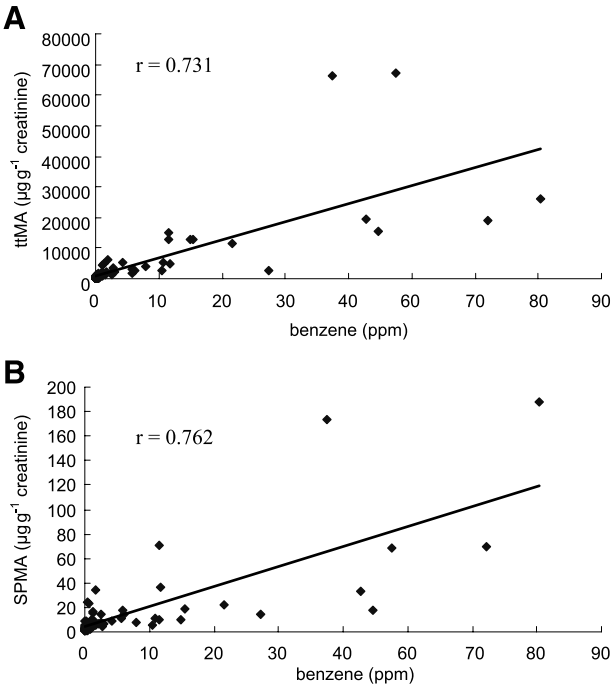

Figure 2. Correlations between benzene exposure levels and its urinary metabolites, ttMA and SPMA, in 70 male benzene-exposed workers. **A,** correlation between benzene exposure level and urinary ttMA level; **B,** correlation between benzene exposure level and urinary SPMA level.

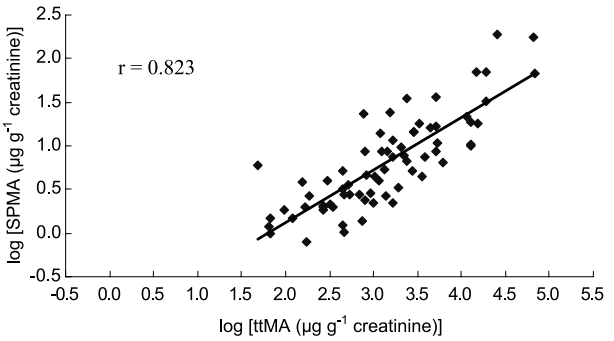

Figure 3. Correlation between urinary ttMA and SPMA levels. These two metabolite levels have been log transformed before statistical correlation analysis.

Effects of *GST* Polymorphism on Benzene Metabolites. The benzene level, ttMA level, SPMA level, and ttMA to SPMA concentration ratio of the population were stratified by *GST* genotype polymorphism and plotted on a logarithmic scale for their distribution, as shown in Fig. 4. To calculate the ttMA to SPMA concentration ratio, the ttMA level was divided by SPMA level in each sample. For age, work seniority, and benzene exposure level, there were no significant differences between two *GSTM1* genotypes. Similar data in *GSTT1* and *GSTP1* were observed (data not shown).

In Fig. 4A, D, and G, no matter in what *GST* genotypes, the urinary ttMA levels showed no significant differences among subgroups of *GSTM1, GSTT1*, and *GSTP1*. In Fig. 4B, the SPMA levels do not show a significant difference in two-tailed *t* test between the two subgroups of *GSTT1*. In several reports (13, 16, 17), lower SPMA levels in *GSTT1*-deficient genotype subjects than in *GSTT1*-positive genotype subjects were reported. It seems that the subjects with *GSTT1*-positive genotype have higher activity of glutathione conjugation. To examine whether the SPMA levels of subjects with *GSTT1*-positive genotype was higher than those of subjects with deficient genotype or not, one-tailed *t* test was adopted. In Fig. 4B, the SPMA levels of subjects with *GSTT1*-positive genotype were significantly higher than those of subjects with *GSTT1*-deficient genotype ($P = 0.041$, one-tailed). The *GSTT1*-positive subjects had 2.2 times higher average urinary SPMA level than the *GSTT1*-deficient subjects. To further show which *GST* genotypes influence the levels of ttMA and SPMA, the concentration ratio of ttMA to SPMA was calculated and classified by the three *GST* genotypes and their subgroups. Significant difference in the ttMA to SPMA concentration ratio was observed between the *GSTT1*-positive and *GSTT1*-deficient genotype groups ($P = 0.002$). The mean ttMA to SPMA concentration ratio in the *GSTT1*-positive group was 1.7 times lower than that in the *GSTT1*-deficient group. The results showed that the subjects with *GSTT1*-positive genotype had higher proportion of SPMA in urine metabolites than those with *GSTT1*-deficient genotype.

As shown in Table 3, the benzene exposure level, ttMA level, SPMA level, and ttMA to SPMA concentration ratio were also stratified by *GST* genotypes and benzene exposure levels (≧1 or <1 ppm). In the high benzene

exposure group (≧1 ppm), only the ttMA to SPMA concentration ratio in the *GSTT1*-positive subjects was significantly higher than that in the *GSTT1*-deficient subjects. In the high benzene exposure group, urinary SPMA level showed no significant difference between *GSTT1*-positive and *GSTT1*-deficient subjects, although the P value (0.176) of one-tailed t test was close to 0.05. In the low benzene exposure group (<1 ppm), for *GSTT1*, both the SPMA level and ttMA to SPMA concentration ratio showed significant differences between positive and deficient genotype subjects. The data showed that the increasing rate of SPMA levels between the two *GSTT1* genotypes in the high exposure group (≧1 ppm) was lower than that in the low exposure group (<1 ppm).

The dose-related productions of ttMA and SPMA versus benzene exposure level were shown in Fig. 5A and B, respectively. The dose-related production of metabolite represents the amount of metabolite generated from the per unit benzene exposure dose. For both metabolites, the regression lines and equations within the two *GSTT1* genotypes are also shown. No matter in what *GSTT1* genotype, the dose-related productions showed downward trends with increasing benzene exposure levels. In the dose-related production of ttMA, the regression lines of the two *GSTT1* genotypes were closely superimposed. There was no apparent difference in dose-related production of ttMA between the two *GSTT1* genotypes. In Fig. 5B, the dose-related production of SPMA in *GSTT1*-positive genotype subjects (1.1 ± 0.79 μg/g creatinine/ppm benzene) was significantly higher than that in *GSTT1*-deficient genotype subjects (0.82 ± 0.68 μg/g creatinine/ppm benzene; $P < 0.05$). In the dose-related production of SPMA, the significant difference between these two regression lines of the two *GSTT1* genotypes was not observed ($P = 0.096$), although the P value was close to 0.05.

The regression lines between ttMA to SPMA concentration ratio and benzene exposure levels for the two *GSTT1* genotypes were shown in Fig. 6. The concentration ratio of ttMA to SPMA reveals the physical production ratio of these two metabolites for biotransformation of benzene into ttMA and SPMA. For both of

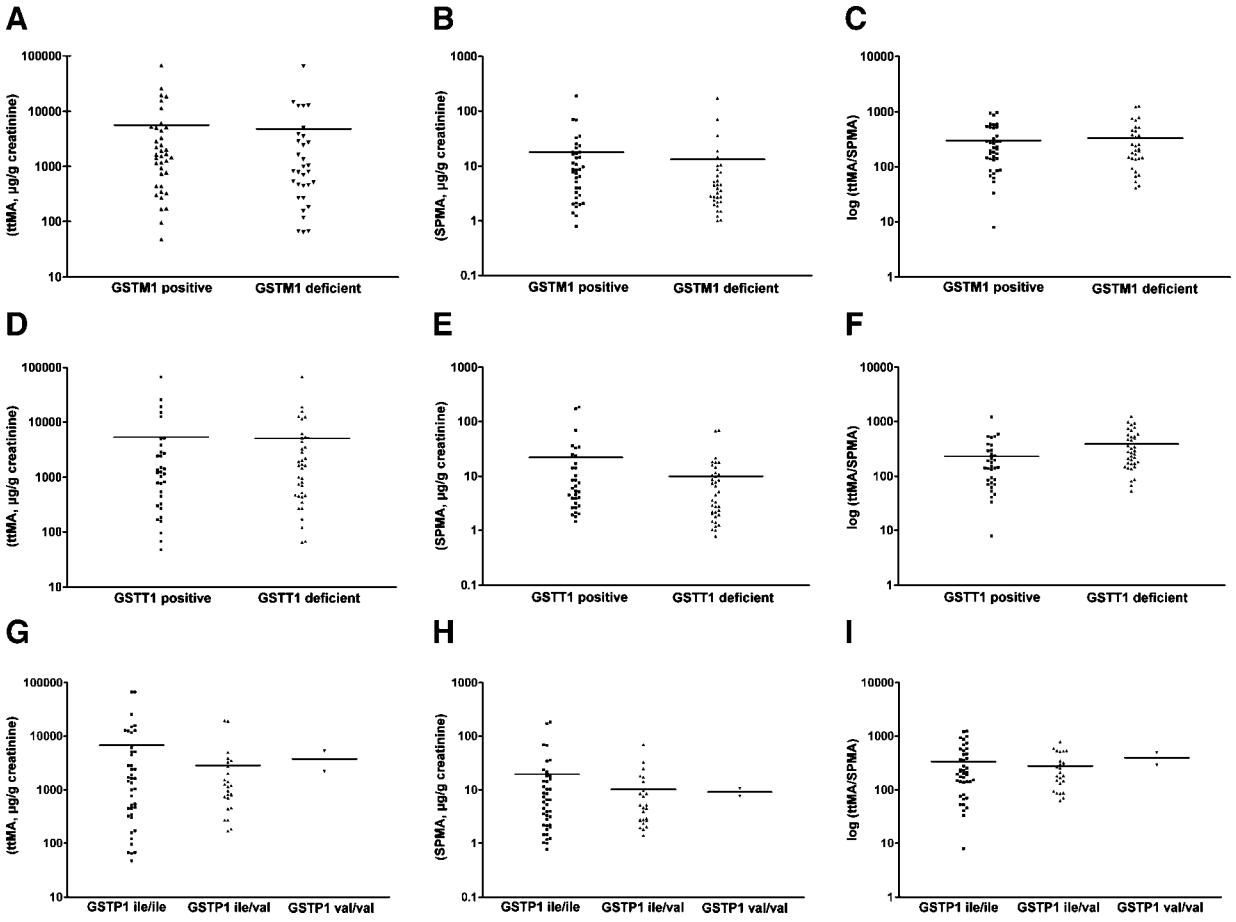

Figure 4. Urinary ttMA levels, SPMA levels, and ttMA to SPMA concentration ratios of whole subjects are plotted by *GST* genotype polymorphism. These variables were statistically tested after log normal transformation. **A** to **F,** variables were tested by independent-sample t test between positive and deficient genotypes in *GSTM1* and *GSTT1*. **G** to **I,** variables were tested by Kruskal-Wallis test among *GSTP1* subgroups. SPMA level and the concentration ratio of ttMA to SPMA showed significant differences between *positive* and deficient genotypes in *GSTT1*. Additionally, the ttMA to SPMA concentration ratio showed more significant difference between *GSTT1* subgroups compared with SPMA level.

Table 3. Airborne benzene, urinary ttMA, SPMA, and ttMA to SPMA concentration ratio stratified by GST genotype polymorphism and benzene exposure level (mean ± SD)

	Variables	Benzene (ppm)	ttMA (μg/g creatinine)*	SPMA (μg/g creatinine)*	ttMA/SPMA*
High benzene exposure (≧1 ppm)	*GSTM1* †				
	Positive (n = 20)	18 ± 23	10,000 ± 15,000	29 ± 42	390 ± 290
	Deficient (n = 13)	10 ± 9.5	11,000 ± 17,000	28 ± 47	520 ± 370
	P	0.898	0.903	0.454	0.232
	GSTT1 †				
	Positive (n = 16)	14 ± 20	11,000 ± 17,000	39 ± 58	340 ± 290
	Deficient (n = 17)	16 ± 19	10,000 ± 16,000	19 ± 20	550 ± 330
	P	0.688	0.566	0.345	0.033
	GSTP1 ‡,§				
	ile/ile (n = 21)	16 ± 19	13,000 ± 19,000	35 ± 52	490 ± 370
	ile/val (n = 10)	14 ± 21	6,100 ± 7,000	19 ± 20	360 ± 230
	val/val (n = 2)	6.7 ± 5.8	3,800 ± 2,200	9.2 ± 2.1	390 ± 150
	P	0.667	0.532	0.543	0.800
Low benzene exposure (<1 ppm)	*GSTM1* †				
	Positive (n = 18)	0.21 ± 0.22	680 ± 550	5.7 ± 7.0	200 ± 160
	Deficient (n = 19)	0.18 ± 0.24	490 ± 400	2.7 ± 1.7	200 ± 180
	P	0.713	0.369	0.123	0.756
	GSTT1 †				
	Positive (n = 17)	0.18 ± 0.22	530 ± 470	5.9 ± 7.1	130 ± 130
	Deficient (n = 20)	0.21 ± 0.23	630 ± 500	2.7 ± 2.1	260 ± 180
	P	0.434	0.448	0.018	0.004
	GSTP1 †,‡				
	ile/ile (n = 21)	0.18 ± 0.26	510 ± 520	3.9 ± 5.0	190 ± 180
	ile/val (n = 15)	0.22 ± 0.19	710 ± 420	4.7 ± 5.8	220 ± 160
	P	0.198	0.054	0.339	0.219

*Data were be statistically tested after log transformation.
† Benzene, ttMA, SPMA, and ttMA/SPMA were tested by independent-sample t test between positive and deficient genotypes in *GSTM1* and GSTT1.
‡ One sample loss in GSTP1.
§ Benzene, ttMA, SPMA, and ttMA/SPMA were tested by Kruskal-Wallis test among GSTP1 subgroups.

the two *GSTT1* genotypes, the regression lines showed a significant upward trend (P < 0.001). The results suggested that the proportion between the urinary metabolites changed with increasing benzene exposure level, no matter in what *GSTT1* genotype. The *GSTT1*-deficient genotype subjects exhibited a higher ttMA to SPMA concentration ratio than the *GSTT1*-positive genotype subjects. The slopes of the two regression lines were also apparently different.

Discussion

Urinary Benzene Metabolites as Exposure Markers. For monitoring benzene exposure levels, the urinary benzene metabolites, ttMA and SPMA, are more widely accepted as exposure biomarkers than other urinary metabolites because of the strong correlation between levels of atmospheric benzene and urinary metabolites (26-28). The ttMA and SPMA levels of urine collected at the end of work-shift have been suggested as BEIs by ACGIH, and the BEI values of ttMA and SPMA were set at 500 and 25 μL g^{-1} creatinine, respectively. However, SPMA was considered to be a better exposure biomarker than ttMA (4, 29). In this study, urinary SPMA and benzene exposure showed a slightly better correlation (r = 0.76, P < 0.05) than did ttMA and benzene exposure (r = 0.73, P < 0.05). Additionally, both ttMA and SPMA showed significant differences between benzene exposure level <1 ppm and benzene exposure level in the range of ≧1 to <10 ppm (P < 0.05; data not shown). The data indicated that the two metabolites could be used to determine benzene exposure level down to 1 ppm (time-weighted average). Several previous reports also showed that urinary ttMA and SPMA are good biomarkers for biomonitoring of low benzene exposure (<1 ppm; refs. 25, 29). The data showed that both the urinary ttMA and SPMA levels collected at the end of work-shift were good benzene exposure biomarkers. According to the high correlation between ttMA and SPMA levels (Fig. 3; r = 0.823), the data indicated that the specificity of urinary ttMA as an exposure marker in these subjects was not heavily interfered with the chemicals known to cause interference, such as sorbic acid (4), a common food preservative.

Association between *GST* Genotype and Two Urinary Benzene Metabolites, ttMA and SPMA. According to the results (Fig. 4; Table 3), the SPMA levels of subjects with *GSTT1*-positive genotype were significantly higher than those of subjects with *GSTT1*-deficient genotype (P = 0.041). Similar observations about the influence of *GSTT1* genotypes on the SPMA level in urine were described in several reports (13, 16, 17). A significant difference in SPMA levels between the two *GSTT1* genotypes was observed in the low exposure group (<1 ppm) but not in the high exposure group (≧1 ppm). The data showed that the increasing rate of SPMA levels between the two *GSTT1* genotypes in the high exposure group (≧1 ppm) was lower than that in the low exposure group (<1 ppm). On the other hand, the increasing rate of SPMA level from *GSTT1*-deficient genotype to *GSTT1*-positive genotype was related to benzene exposure level. These results reveal that SPMA level was associated not only with *GSTT1* genetic polymorphism but also with benzene exposure level.

The concentration ratio of ttMA to SPMA reveals the physical production ratio of these two metabolites for

biotransformation of benzene into ttMA and SPMA, assuming that the excretion efficiencies of these two metabolites are constant. No matter in what benzene exposure subgroup (Table 3), a significant difference in ttMA to SPMA ratio between the two *GSTT1* genotypes was also observed. The data reveal that the subjects with *GSTT1*-positive genotype had a higher proportion of SPMA in the two urinary metabolites than those with *GSTT1*-deficient genotype.

As shown in Tables 2 and 3, the SDs of ttMA and SPMA were large. One of the limitations in the study is number of subjects (n = 70). In Table 3, there seems to be a trend of decreasing ttMA and SPMA with *GSTP1* genotype in the high benzene exposure group, although two metabolite levels showed no statistically significant difference among the three *GSTP1* genotypes. For observation of the association between urinary benzene metabolites and *GSTP1* genetic polymorphism, the larger sample number should be required.

According to Fig. 5A, urinary ttMA displayed a downward trend of dose-related production with increasing benzene exposure levels. A similar observation was also described in a previous report (18). The downward trend of dose-related production of both benzene metabolites is considered to be related to a saturable enzymatic metabolic pathway. For benzene

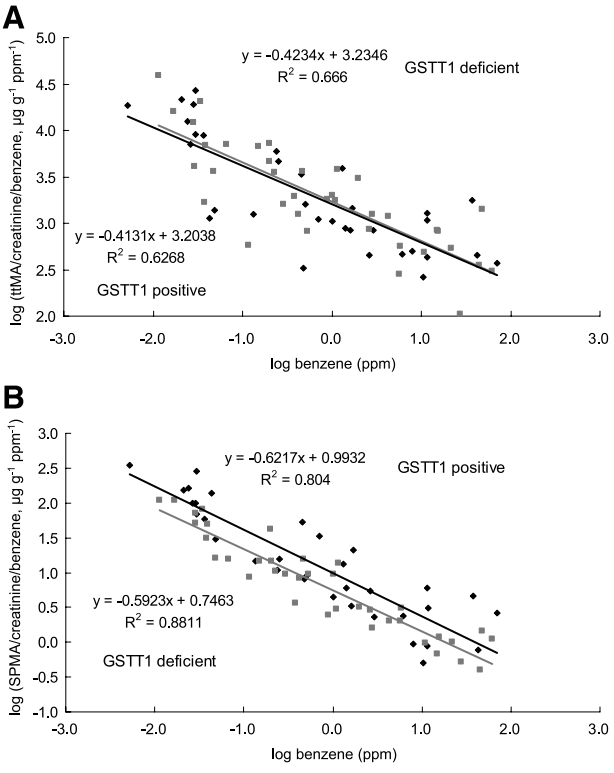

Figure 5. The correlations between dose-related productions of metabolites, ttMA (**A**) and SPMA (**B**), and benzene exposure levels are plotted by the two *GSTT1* genotypes. The black-diamond plot and gray-square plot represent the *GSTT1*-positive and *GSTT1*-deficient genotypes, respectively. The black and gray regression lines represent the *GSTT1*-positive and *GSTT1*-deficient genotypes, respectively.

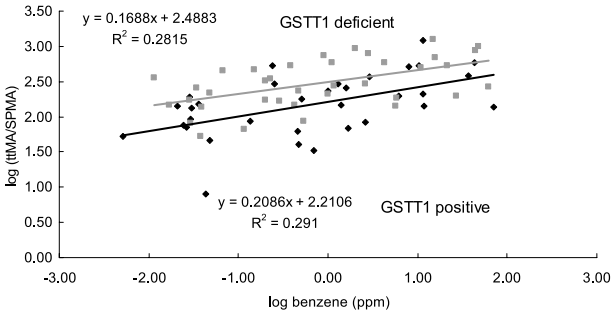

Figure 6. The correlations between ttMA to SPMA concentration ratio and benzene exposure levels are plotted by the two *GSTT1* genotypes. The black-diamond plot and gray-square plot represent the *GSTT1*-positive and *GSTT1*-deficient genotypes, respectively. The black and gray regression lines represent the *GSTT1*-positive and *GSTT1*-deficient genotypes, respectively.

metabolism, the oxidative reaction of CYP2E1 was considered as a major saturable metabolic stage (18). Comparing the trend of dose-related production of SPMA described in a report of Kim et al. (18) with our data, the observations on the trend were not consistent. In Fig. 5B, urinary SPMA displayed a downward trend of dose-related production. The same phenomenon of SPMA production by calculating the dose-related production from a report of Melikian et al., was also observed (29). In the report of Melikian et al., the trend of SPMA dose–related productions in benzene-exposed workers showed that SPMA was related to a saturable enzymatic metabolic pathway. According to our data, both ttMA and SPMA might be related to saturable enzymatic metabolic pathways.

Taking together the data in Fig. 5, no matter in what *GSTT1* genotype, the dose-related production of ttMA and SPMA showed a downward trend with increasing benzene exposure level. The ttMA levels were not associated to *GSTT1* genotypes, whereas their regression lines were closely superimposed as shown in Fig. 5A. Comparing the data between the two *GSTT1* genotypes, the dose-related production levels of SPMA were higher in *GSTT1*-positive genotype subjects ($P < 0.05$). A similar phenomenon in the report of Qu et al. (16) was observed. Additionally, with increasing benzene exposure level, the dose-related production of SPMA for *GSTT1*-positive genotype decreased more rapidly than that for *GSTT1*-deficient genotype. The data showed that SPMA dose-related production was associated not only with *GSTT1* genetic polymorphism but also with benzene exposure level. However, no correlation between *GSTT1* genetic polymorphism and dose-production of ttMA was observed.

In Fig. 6, the regression lines from ttMA to SPMA ratios versus benzene exposure levels for the two *GSTT1* genotypes were shown. The ttMA to SPMA ratios in *GSTT1*-deficient subjects were higher than those in *GSTT1*-positive subjects. For both of these two *GSTT1* genotypes, the ttMA to SPMA ratios increased with the benzene exposure levels, indicating that the proportion of ttMA in the two urinary metabolites increased with benzene exposure levels. That is, the proportions of a

urinary benzene metabolite could change with the benzene exposure levels. The data showed that the increasing rate of ttMA to SPMA ratio from *GSTT1*-positive genotype to *GSTT1*-deficient genotype decreased with increasing benzene exposure levels. Similar results were also shown in Table 3. These data seem to indicate that the influence of *GSTT1* genetic polymorphism on ttMA to SPMA ratio decreased with increasing benzene exposure level.

According to the BEI values of ttMA (500 μg g^{-1} creatinine) and SPMA (25 μg g^{-1} creatinine) from ACGIH, the BEI ratio of ttMA to SPMA is 20. The BEI values of ttMA and SPMA were determined based on western population described in four reports (25, 27, 30, 31) from ACGIH. In our data from Taiwan population, the ttMA to SPMA ratio in the low benzene exposure group (<1 ppm) was 144. In a previous report of China population, the ttMA to SPMA ratio in the high benzene exposure group (≧1 ppm) was 40 by calculating the ttMA and SPMA mean values of the post-work (29). According to our data and the report of Qu et al., the ttMA to SPMA ratio of China population was >20.

According to our observations and previous reports (13, 16, 17), the *GSTT1* genetic polymorphism influences the concentration ratio of ttMA to SPMA. However, the different races of populations with different *GSTT1* allele frequencies were reported (23). *GSTT1* allele frequencies, including positive and deficient genotypes, were 40% and 60%, respectively, in a previous report in China/Taiwan population (23). In Caucasian population, including Europeans and Caucasian Americans, *GSTT1*-positive and *GSTT1*-deficient allele frequencies were 82.5% and 17.5%, respectively (23). By comparing the *GSTT1* genotype frequencies between the China/Taiwan and Caucasian populations, the population in China/Taiwan seems to have higher ttMA to SPMA ratio than the Caucasian population. The difference in ttMA to SPMA ratio between our data and ACGIH may be related to different races with different *GSTT1* frequencies. However, the increasing level of ttMA to SPMA ratio between the two different races should be further investigated. The results also suggest that the BEI values should be set for populations of different races.

Several studies that have reported on the ttMA and SPMA levels relating to *GST* polymorphism were listed in Table 4. Among these reports, *GSTP1* was not associated with the urinary levels of ttMA and SPMA. Three studies showed that subjects with the *GSTT1*-positive genotype had significantly higher SPMA excretion levels than did subjects with *GST*-deficient genotype (13, 16, 17). The study of Bergamaschi and colleagues (15) showed that subjects with *GSTM1*-deficient genotype had significantly higher ttMA levels than subjects with *GSTM1*-deficient genotype. It is the only one report showing that ttMA is associated with *GSTM1* genetic polymorphism in 24 subjects. Surprisingly, there was no association between SPMA level and *GSTM1* genetic polymorphism. However, ttMA could be reliable in detecting benzene exposure level down to 1 ppm (4, 25). The benzene exposure level in the study that ranged from 0.007 to 0.026 ppm with a median of 0.015 ppm was below the cutoff level (1 ppm). Especially, ttMA could be interfered with the common food preservative (4). Therefore, conclusions from the study have to be cautiously used. Another study by Rossi and his colleagues (14) showed that significantly higher ttMA excretion levels were found in *GSTT1*-deficient subjects than in *GSTT1*-positive subjects at the benzene exposure level of 0.026 ppm. Additionally, there was a study that showed that no association between urinary benzene metabolite level and *GST* genetic polymorphism was observed at the benzene exposure level of 0.78 ppb (32). However, the low benzene exposure level in these reports is still a problem.

Hydroquinone is a benzene metabolite with myelotoxin and is reported to be associated with micronuclei,

Table 4. GST polymorphism related to the levels of benzene metabolites in literature

Population subject characteristic (*n*), city	GST genotypes related to the levels of benzene urinary metabolites			Description	Reference
	GSTM1	*GSTT1*	*GSTP1*		
Taxi driver (29)	Not related to metabolite level	SPMA	Not related to metabolite level	Lower SPMA level in *GSTT1*-deficient genotype	(13)
Roadside resident (37)					
Suburban (42)					
Rural (40), Cotonou					
Oil shale mine surface worker (50)	Not related to metabolite level	SPMA	Not related to metabolite level	Lower SPMA level in *GSTT1*-deficient genotype	(17)
Oil shale mine underground worker (50), Estonian					
Exposed worker (130)	Not related to metabolite level	SPMA	—	Lower SPMA level in *GSTT1*-deficient genotype	(16)
Unexposed worker (51), China					
Bicycle driver (24), Italy	ttMA	Not related to metabolite level	—	Higher ttMA level in *GSTM1*-deficient genotype	(15)
Bus driver (59), Bologna	Not related to metabolite level	ttMA	Not related to metabolite level	Higher ttMA level in *GSTT1*-deficient genotype	(14)
Resident (40), Copenhagen	Not related to metabolite level	Not related to metabolite level	Not related to metabolite level	—	(32)
Worker (70), Taiwan	Not related to metabolite level	SPMA	Not related to metabolite level	Lower SPMA level in *GSTT1*-deficient genotype	This study

sister chromatid exchange, and chromosomal aberrations (9). The association between *GSTM1* genetic polymorphism and DNA damage caused by hydroquinone in human lymphocytes was reported. However, *GSTT1* genotype was not related to any DNA damage in human lymphocytes (10). Furthermore, both *GSTM1* and *GSTT1* were related to low WBC in benzene-exposed workers (10). These data showed that the *GSTM1* genetic polymorphism is related to several health effects caused by benzene. However, in our data, *GSTM1* genotype was not related to the levels of the urinary benzene metabolites. Furthermore, the SPMA levels of *GSTM1*-positive subjects in the low benzene exposure group (<1 ppm) were higher than those of *GSTM1*-deficient subjects, although there was no significant difference between the two *GSTM1* genotypes (Table 3). If the sample size could be increased, the effects of *GSTM1* genetic polymorphism on benzene metabolism could apparently be observed.

Conclusion

Among the *GST* genotypes investigated in this study, the results showed that only the *GSTT1* genotype was related to the level and dose-related production of SPMA. The subjects with *GSTT1*-positive genotype had higher levels of SPMA than those with *GSTT1*-deficient genotype. Associations between benzene exposure level and dose-related productions of ttMA and SPMA were reported. Additionally, the *GSTT1* genetic polymorphism related to dose-related production was further shown. According to our data, no matter in what *GSTT1* genotypes, the proportion of ttMA in urine increased with benzene exposure level by observation of ttMA to SPMA ratio versus benzene exposure level. Furthermore, the proportions of urinary benzene metabolites changed with exposure levels. Using SPMA for evaluating benzene exposure, the results suggest that the *GSTT1* genetic polymorphism, especially in a comparison study between two populations with different *GSTT1* genotype frequencies, should be considered. Additionally, the BEI value of SPMA should be set based on the levels of subjects with *GSTT1*-deficient genotypes for protection of all subjects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. International Agency for Research on Cancer (IARC). Monographs on the evaluation of carcinogenic risk of chemicals to humans. Chemicals, industrial processes and industries associated with cancer in humans. Lyon: IARC; 1987.
2. Ong CN, Lee BL. Determination of benzene and its metabolites: application in biological monitoring of environmental and occupational exposure to benzene. J Chromatogr B Biomed Appl 1994;12:1–22.
3. Yardley-Jones A, Anderson D, Parke DV. The toxicity of benzene and its metabolism and molecular pathology in human risk assessment. Br J Ind Med 1991;48:437–44.
4. Dor F, Dab W, Empereur-Bissonnet P, Zmirou D. Validity of biomarkers in environmental health studies: the case of PAHs and benzene. Crit Rev Toxicol 1999;29:129–68.
5. American Conference of Governmental Industrial Hygienists (ACGIH). Documentation of the biological exposure indices. 7th ed. Benzene. 2001; Benzene BEI1-Benzene BEI14.
6. Strange R, Jones P, Freyer A. Glutathione *S*-transferase: genetics and role in toxicology. Toxicol Lett 2000;112–113:357–63.
7. Watsons MA, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione *S*-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis 1998;19:275–80.
8. Xu X, Wiencke JK, Niu T, et al. Benzene exposure, glutathione *S*-transferase θ homozygous deletion, and sister chromatid exchanges. Am J Ind Med 1998;33:157–63.
9. Silva MC, Gaspar J, Silva ID, Faber A, Rueff J. GSTM1, GSTT1, and GSTP1 genotypes and the genotoxicity of hydroquinone in human lymphocytes. Environ Mol Mutagen 2004;43:258–64.
10. Hsieh LL, Liou SH, Chiu LL, Chen YH. Glutathione *S*-transfrase (GST) M1 and GSTT1 genotypes and hematopoietic effects of benzene exposure. Arch Toxicol 1999;73:80–2.
11. Wan J, Shi J, Hui L, et al. Association of genetic polymorphisms in CYP2E1, MPO, NQO1, GSTM1, and GSTT1 genes with benzene poisoning. Environ Health Perspect 2002;110:1213–8.
12. Wang X, Chen D, Niu T, et al. Genetic susceptibility to benzene and shortened gestation: evidence of gene-environment interaction. Am J Epidemiol 2000;152:693–700.
13. Avogbe PH, Ayi-Fanou L, Autrup H, et al. Ultrafine particulate matter and high-level benzene urban air pollution in relation to oxidative DNA damage. Carcinogenesis 2005;26:613–20.
14. Rossi AM, Guarnieri C, Rovesti S, et al. Genetic polymorphisms influence variability in benzene metabolism in humans. Pharmacogenetics 1999;9:445–51.
15. Bergamaschi E, Brustolin A, Palma CD, et al. Biomarkers of dose and susceptibility in cyclists exposed to monoaromatic hydrocarbons. Toxicol Lett 1999;108:241–7.
16. Qu Q, Shore R, Li G, et al. Biomarkers of benzene: urinary metabolites in relation to individual genotype and personal exposure. Chem Biol Interact 2005;153–154:85–95.
17. Sørensen M, Poole J, Autrup H, et al. Benzene exposure assessed by metabolite excretion in Estonian oil shale mineworkers: influence of glutathione *S*-transferase polymorphisms. Cancer Epidemiol Biomarkers Prev 2004;13:1729–35.
18. Kim S, Vermeulen R, Waidyanatha S, et al. Using urinary biomarkers to elucidate dose-related patterns of human benzene metabolism. Carcinogenesis 2006;27:772–81.
19. Lin LC, Chiung YM, Shih JF, Shin TS, Liao PC. Validation of an online dual-loop cleanup device with an electrospray ionization tandem mass spectrometry-based system for simultaneous quantitative analysis of urinary benzene exposure biomarkers *trans*,*trans*-muconic acid and *S*-phenylmercapturic acid. Anal Chim Acta 2006;555:34–40.
20. Dirksen U, Moghadam KA, Mambetova C, Esser C, Führer M, Burdach S. Glutathione S transferase θ1 gene (GSTT1) null genotype is associated with an increased risk for acquired aplastic anemia in children. Pediatr Res 2004;55:466–71.
21. Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione *S*-transferase π locus association with susceptibility to bladder, testicular and cancer. Carcinogenesis 1997;18:641–4.
22. Tsutakawa RK, Hewett JE. Comparison of two regression lines over afinite interval. Biometrics 1978;34:391–8.
23. Kiffmeyer WR, Langer E, Davies SM, Envall J, Robison LL, Ross JA. Genetic polymorphisms in the Hmong population. Cancer 2004;100:411–7.
24. Tan W, Song N, Wamg G-Q, et al. Impact of Genetic Polymorphisms in cytochrome *P*450 2E1 and glutathione *S*-transferases M1, T1, and P1 on susceptibility to esophageal cancer among high-risk individuals in China. Cancer Epidemiol Biomarkers Prev 2000;9:551–6.
25. Boogaard PJ, van Sittert NJ. Biological monitoring of exposure to benzene: a comparison between *S*-phenylmercapturic acid, *trans*, *trans*-muconic acid, and phenol. Occup Environ Med 1995;52:611–20.
26. Ghittori S, Maestri L, Fiorentino ML, Imbriani M. Evaluation of occupational exposure to benzene by urinalysis. Int Arch Occup Environ Health 1995;67:195–200.
27. Popp W, Rauscher D, Muller G, Angerer J, Norpoth K. Concentrations

of benzene in blood and *S*-phenylmercapturic and *t*,*t*-muconic acid in urine in car mechanics. Int Arch Occup Environ Health 1994;66: 1–6.

28. Boogaard PJ, Van Sittert NJ. Suitability of S-phenyl mercapturic acid and *trans*,*trans*-muconic acid as biomarkers for exposure to low concentrations of benzene. Environ Health Perspect 1996;104: 1151–7.
29. Melikian AA, Qu Q, Shore R, et al. Personal exposure to different levels of benzene and its relationships to the urinary metabolites *S*-phenylmercapturic acid and *trans*,*trans*-muconic acid. J Chromatogr B Biomed Appl 2002;778:211–21.
30. Van Sittert NJ, Boogaard PJ, Beulink GDJ. Application of the urinary *S*-phenylmercapturic acid test as a biomarker for low levels of exposure to benzene in industry. Br J Ind Med 1993;50: 460–9.
31. Ghittori S, Maestri L, Florentino ML, Imbriani M. Evaluation of occupational exposure to benzene by urine analysis. Int Arch Occup Environ Health 1995;67:195–200.
32. Sørensen M, Skov H, Autrup H, Hertel O, Loft S. Urban benzene exposure and oxidative DNA damage: influence of genetic polymorphisms in metabolism genes. Sci Total Environ 2003;309: 69–80.