Are polymorphisms in metabolism protective or a risk for reduced white blood cell counts in a Chinese population with low occupational benzene exposures?

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Background: Genetic variations in metabolic enzyme genes may enhance hematotoxicity in benzene-exposed populations.

Objective: To investigate the association between polymorphisms of metabolism genes and white blood cells (WBCs).

Methods: Three hundred and eighty-five benzene-exposed workers and 220 unexposed indoor workers were recruited in China. We explored the relationship between metabolic enzymes polymorphisms [*glutathione S-transferase T1/M1* (*GSTT1/M1*) null, *glutathione S-transferase P1* (*GSTP1*)rs1695, *Cytochrome P450 2E1* (*CYP2E1*) rs3813867, rs2031920, rs6413432, microsomal epoxide hydrolase (mEH) rs1051740, rs2234922] by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis and WBC.

Results: The exposed group had lower WBC counts ($P < 0.001$) than the unexposed group. Increased susceptibility to hematotoxicity, as evidenced by lower WBC counts, was found in workers with null-*GSTT1* ($P = 0.045$), null-*GSTM1* ($P = 0.030$), rs2031920 ($P = 0.020$), and rs3813867 ($P = 0.014$) genotypes. White blood cell counts were also lower in workers with null-*GSTT1* and null-*GSTM1* after adjusting for age, gender, smoking, and alcohol consumption.

Conclusion: Null-*GSTT1* and null-*GSTM1* genotypes and *Cytochrome P4502E1* (*CYP2E1*: rs2031920, rs3813867) may support the hematotoxicity of benzene-exposed workers in China, and we can make use of it to select susceptible population.

Keywords: Benzene, White blood cell, Genetic polymorphism, CYP2E1, GSTs, mEH

Introduction

Benzene is an important industrial organic solvent and a common component of gasoline, engine exhaust, wood smoke, and tobacco smoke. It is ubiquitous in the environment and an established hematotoxic and genotoxic carcinogen (IARC, 1982). Epidemiological evidence indicates that benzene exposure is associated with hematotoxicity, hematopoietic dysfunction, and the development of aplastic anemia and leukemia.^{1–3} What was once considered a

low exposure (less than 1 ppm of benzene as per the U.S. occupational guidelines for allowable exposure limits) has been linked to reductions in white blood cell (WBC) counts.⁴

Although the exact mechanisms of benzene-induced hematotoxicity and genotoxicity remain unclear and controversial, it is believed that benzene itself has no mutagenic or cytotoxic activity, instead acting through a series of benzene metabolites.⁵ Researchers have found that benzene metabolites may be singularly, collectively, directly, or indirectly implicated in the progression of benzene-related toxicity.^{5,6} After inhalation, benzene is oxidized in the liver by CYP2E1 to form benzene oxide, an

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electrophile that binds to macromolecules and the source of all other metabolites.⁷ Spontaneous rearrangement of benzene oxide produces phenol (PH), which can undergo another CYP oxidation, resulting in potentially toxic hydroquinone (HQ).^{8,9} Benzene oxide can also be hydrolyzed by microsomal epoxide hydrolase (mEH) into benzene dihydrodiol that is then converted to a catechol. A separate possibility is for the benzene oxide to undergo ring opening to produce *trans*, *trans*-mucoaldehyde, which can spontaneously rearrange to form PH, which is hydroxylated in the liver to form HQ.⁹ Catalyzed by phase II metabolic enzymes, such as glutathione S-transferase (GST), these benzene metabolites are easily conjugated with glutathione or glucuronide to form less toxic or non-toxic derivatives^{10,11} excreted in urine. In addition, the hematotoxic and leukemogenic metabolite of benzene, 1,4-benzoquinone (1,4-BQ), reacts rapidly with macromolecules such as albumin. We hypothesized that the deficient or altered phase I (*CYP2E1*, *mEH*) and phase II (*GSTs*) metabolic enzymes involved in benzene metabolism affect individual susceptibility to benzene toxicity.

Genetic polymorphisms in genes encoding *CYP2E1*, *mEH*, and *GSTs* may account for human variable responses to benzene because they affect the level of expression, structure, or catalytic activity of these metabolic enzymes.¹² Genetic predisposition (polymorphisms) to benzene toxicity is currently under investigation.^{13–15} Previous studies have explored the relationship between genetic polymorphisms and benzene metabolism on the premise of high exposure, or without precise measurements of air exposure, or with small numbers of participants.¹³ The overall effects of polymorphism sites in some of the genes are unclear, especially the study of the promoter region of *CYP2E1*, which has yielded mixed results.¹³ This paper explores the relationship of genes with benzene exposure and blood cell counts. We adopted WBC counts as the biomarker and used the PCR–restriction fragment length polymorphism (RFLP) technique to determine gene polymorphisms to evaluate the susceptibility of individuals with different metabolic enzyme genetic polymorphisms to benzene-induced hematotoxicity.

Methods

Study population

The Research Ethics Review Board of the School of Public Health at Fudan University approved this study protocol, and written informed consent was obtained from all participants. Benzene-exposed workers were recruited from six shoe factories during routine medical evaluations in Zhejiang Province, China. All six factories have similar manufacturing techniques. The 385 workers (190 males and 195

females, aged 19–57 years) were directly exposed to benzene for at least one year. Interviews were completed to collect the following variables: age, job category, occupational history, smoking, alcohol consumption, daily work hours, and individual and family medical histories. An additional 220 healthy workers (127 men and 93 women) were recruited from the same city to be the unexposed controls. Teachers and bank clerks were matched by gender and age (19–57 years). Excluded were subjects with recent exposure to X-ray or with an infection. Exposed and unexposed workers were enrolled from August 2011 to March 2013. Trained staff interviewed each participant. Anti-coagulated blood samples were collected from all workers as part of a routine medical evaluation.

Benzene exposure assessment

Each shoe factory has three primary workstations: sewing, molding, and packing. Glue brushing, which involves brushes being manually dipped into open containers, was common in all three areas. It is also a source of benzene exposure via inhalation. Benzene concentrations were measured at the breathing level of workers by point sampling. Active carbon tubes were used to collect vapor samples according to the national standard of China (GBZ 159-2004).¹⁶ Samples were collected three times a day (9:00, 11:00, and 16:00) with 0.1 l/min for 15 minutes using an air sampler GS – IIIB (Hongyu, Shanghai, China). Testing was performed using the standard aromatic hydrocarbon compound detection method with gas chromatography, in line with national Chinese standards (CBZ/T160.42-2007).¹⁷ Calculation of the cumulative exposure dose (CED) for workers was based on their work history, work location, and length of employment. The geometric mean of benzene concentration *C* was also estimated for each worksite. The CED was calculated as $CED (mg/m^3 \cdot year) = \sum C (mg/m^3) \times T (year)$, according methodology by Wang *et al.*¹⁸

White blood cell counts

White blood cell count is a compulsory component of the medical examination for workers exposed to benzene. An automated hematology analyzer (XE-2100, Sysmex, Japan) detected WBC counts of both the exposed and the unexposed groups.

DNA extraction and single nucleotide polymorphism genotyping

Genomic DNA was isolated from peripheral blood using a Life DNA isolation kit (Lifefeng Biotechnology Co, Shanghai, China) according to the manufacturer's directions, and frozen at $-80^{\circ}C$. All restricted enzymes were bought from Fermentas (Fermentas, Burlington, Ontario, Canada). Approximately 50 ng of genomic DNA was amplified

in GeneAmp 9600 (Perkin Elmer Corp., Waltham, MA, USA) in a total volume of 15 µl consisting of 0.4 µl for each primer, 7.5 µl of 2× PCR Mix, and 5.7 µl of ddH₂O, as previously described by Wang *et al.*¹⁹ Eight sites involved in benzene phase I and II metabolic enzymes [*GSTT1* null,²⁰ *GSTM1* null,²⁰ *GSTP1 EX5 A>G* (rs 1695), *CYP2E1 -1019 C>T* (rs 2031920),²¹ *CYP2E1 -1293 G>C* (rs 3813867),²⁰ *CYP2E1 Intro6 T>A* (rs 6413432),²² *mEH EX3 3 T>C* (rs 1051740), *mEH EX 4 A>G* (rs 2234922)] were determined by PCR and RFLP analysis. The genes studied and the respective primers and restricted enzymes are provided in Table 1. For each genotype RFLP method, 10% of the positive and negative samples were randomly selected for DNA sequencing to verify the accuracy of the method.

Statistical analysis

Statistical Analysis System (SAS, Version 9.1) software was used for data analysis. Student's *t*-tests were used to compare differences between continuous variables, including age, and CED. White blood cells were evaluated using normality tests. One-way ANOVA was used to detect differences in WBC counts among the exposure groups. The Dunnett test compared the exposed groups with unexposed separately. Hardy-Weinberg equilibrium was tested for each polymorphism, and the observed genotype frequencies were compared to the expected frequencies using linear-regression analysis after adjusting for gender, smoking, drinking, and age. The criterion for significance was set at $P<0.05$. Multiple linear regression analyzed the risk factors and genotypes for benzene-exposed workers. PHASE software (version

2.0.2) was used to obtain maximum-likelihood estimates of the CYP2E1 and mEH diplotype frequencies as previously described.²³

Results

Benzene exposure assessment

Benzene was detected at all of the shoe factories, with an 8 h time-weighted average (TWA) concentration range from 2.6 to 57.0 mg/m³ (median, 6.4 mg/m³). Fifteen of 26 monitoring sites exceeded the national airborne benzene standard in China (6.00 mg/m³). Each benzene-exposed worker's CED was calculated according to the job site, employment duration, and work history, and values ranged from 5.02 to 1183.34 mg/m³-year, with a median at 32.19 mg/m³-year. The exposed workers were divided into four groups (≥ 5.02 , >19.90 , >31.81 , and >59.00 mg/m³-year) by quarter CED. The exposed workers were also divided into three groups based on benzene concentrations according to the U.S. (1 ppm, equal to 3.25 mg/m³) and China's standard (6.00 mg/m³).

WBC counts and its variation by demographics and life styles

Table 2 shows the distribution of socio-demographic variables in the exposed and unexposed, and their association with WBC counts. Linear regression indicated that WBCs of older workers (>30 vs ≤ 30 years; 5.33 ± 1.37 vs 5.81 ± 1.69 , $P=0.003$) in the exposed group were lower than in the younger age group. Somewhat surprisingly, smokers had higher WBC counts than the non-smokers (6.01 ± 1.89 vs 5.48 ± 1.45 , respectively, $P=0.005$).

Table 1 Primers and enzymes used for PCR-RFLP genotyping

Gene (polymorphism)	ID	Primers	Restriction enzyme	Banding pattern
<i>GSTM1</i>	–	GAAGTCCCTGAAAAGCTAAAGC GTTGGGCTCAAATATACGGTGG	–	219 bp
<i>GSTT1</i>	–	TTCCTTACTGGTCCTCACATCTC TCACCGGATCATGGCCAGCA	–	459 bp
<i>ALB</i>	–	GCCCTCTGCTAACAAGTCCTAC GCCCTAAAAAGAAAATCGCCAATC	–	350 bp
<i>CYP2E1 -1293 G>C</i>	rs3813867	CAGTCGAGTCTACATTGTC TTCATTCTGTCTTCTAACTG	RsaI	GG: 410 bp GC: 410, 290, 120 bp CC: 290, 120 bp
<i>CYP2E1-1019 C>T</i>	rs2031920	CCCGTGAGCCAGTCGAGT ATACAGACCCTCTTCCAC	PstI	CC: 510 bp CT: 510, 360, 150 bp TT: 150, 360 bp
<i>CYP2E1 intro6 T>A</i>	rs6413432	CTGCTGCTAATGGTCACTTG GGAGTTCAAGACCAGCCTAC	Hinfi	TT: 688 bp TA: 688, 350, 338 bp AA: 350, 338 bp
<i>GSTP1 EX5 A>G</i>	rs1695	CTTCCACGCACATCCTCTTCC AAGCCCCCTTTCTTTGTTTCAGC	Alw26I	Ile/Ile(AA):289 bp Val/Val (GG): 218, 71 bp Ile/Val (AG): 289, 218, 71 bp
<i>mEH EX3-28T>C</i>	rs1051740	GATCGATAAGTTCCGTTTCACC ATCCTTAGTCTTGAAGTGAGGAT	EcoR V	His/His(TT): 140, 22 bp Tyr/Tyr (CC):162 bp His/Tyr (CT): 162, 140, 22 bp
<i>mEH EX4 +52A>G</i>	rs2234922	ACATCCACTTCATCCACGT ATGCCTCTGAGAAGCCAT	RsaI	His/His (AA):210 bp Arg/Arg(GG):164, 46 bp His/Arg (AG): 210, 64, 46 bp

Table 2 Summary of WBC counts and their variation with risk factors by benzene exposure

Group	Number	Mean WBC ^a ±SD (×10 ⁹)	t	P
Control				
Gender				
Male	127	6.62±1.33	1.984	0.483
Female	93	6.24±1.48		
Age (years)				
≤30	144	6.58±1.46	1.722	0.167
>30	76	6.24±1.27		
Smoking				
Non-smoker	193	6.45±1.39	0.377	0.702
Smoker	27	6.56±1.52		
Alcohol				
Non-user	162	6.43±1.35	0.603	0.229
User	58	6.56±1.56		
Exposure				
Gender				
Male	190	5.75±1.66	1.692	0.091
Female	195	5.47±1.48		
Age (years)				
≤30	224	5.81±1.69	2.975	0.003
>30	161	5.33±1.37		
Smoking				
Non-smoker	292	5.48±1.45	-2.850	0.005
Smoker	93	6.01±1.89		
Drinking				
Non-user	249	5.62±1.51	0.194	0.864
User	136	5.59±1.70		

^a WBC, white blood cell; Results derived from independent-samples *t* test.

Correlation between benzene CED and WBC counts

The mean WBC counts decreased by benzene CED and concentrations in workers and are presented in Table 3. In the exposed group, mean WBC counts (5.61 ± 1.58) were lower than in the unexposed group (6.47 ± 1.40) ($P < 0.01$). One-way ANOVA showed a statistically significant decrease in WBC in relation to cumulative benzene exposure ($P < 0.01$) and concentration ($P < 0.01$).

Prevalence of genotypes in metabolic enzyme genes among the exposed workers

Table 4 shows the frequencies of *GSTT1*, *GSTM1*, *GSTP1* exon 5, *CYP2E1* -1019, *CYP2E1* -1293, *CYP2E1* Intro6, *mEH* exon 3, *mEH* exon 4 genotypes and their association with WBC counts. All the genotypes conformed to Hardy–Weinberg equilibrium. The WBC counts in *GSTT1*-null (null vs present; 5.42 ± 1.45 vs 5.81 ± 1.68 , $P = 0.045$) and *GSTM1*-null (null vs present; 5.43 ± 1.45 vs 5.75 ± 1.67 , $P = 0.030$) genotypes were significantly decreased compared to the *GSTT1*/M1 present group after adjustment for gender, smoking, and alcohol consumption. There were lower WBC counts for individuals who possessed the *CYP2E1* (rs2031920) CT genotype compared with the CC genotype (mean±SD: 5.35 ± 1.43 vs 5.75 ± 1.63 , $P = 0.020$). *CYP2E1* (rs3813867) GC genotype was also associated with a significantly lower WBC count compared with the GG genotype (mean±SD: 5.36 ± 1.30 vs 5.74 ± 1.69 , $P = 0.020$). There was no significant relationship between the WBC and variant alleles in *mEH* 3 (rs1051740) (variant alleles CC vs wild-type TT: 5.78 ± 1.63 vs 5.54 ± 1.54 , $P = 0.197$) and *mEH* 4 (rs2234922) (variant alleles GG vs wild-type AA: 5.35 ± 1.07 vs 5.60 ± 1.49 , $P = 0.910$). The WBC in predicted fast *mEH* activity (fast vs slow: 4.85 ± 1.21 vs 5.64 ± 1.60 , $P = 0.166$) was higher than slow group, although the difference was not statistically significant.

The effect on WBCs by metabolic enzymes polymorphisms within the CED in a multiplicative model for benzene-exposed workers is shown in Table 5. At the highest level (59.00–364.11 mg/m³), the WBCs in null-*GSTM1* group decreased further

Table 3 The white blood cell counts at various exposure groups

Subjects	Number	Mean WBC±SD (×10 ⁹)
Unexposed	220	6.47±1.40
Benzene-exposed CED (mg/m ³ -year) ^a		
≥5.02	96	6.17±1.58
>19.90	96	5.63±1.54**
>31.81	96	5.45±1.81**
>59.00	97	5.19±1.20**
F ^c		18.86
P ^c		<0.001
Benzene-exposed concentration (mg/m ³) ^b		
<3.25	24	5.57±1.79*
<6.00	149	6.01±1.47
≥6.00	212	5.27±1.54**
F ^c		17.04
P ^c		<0.001
Exposed	385	5.61±1.58**
F ^c		24.46
P ^c		<0.001

^aThe exposed workers were divided into four groups with quarter cumulative exposure dose (CED).

^b The exposed workers were divided into three groups by benzene concentrations according to US (1 ppm, equal to 3.25 mg/m³) and China standards (6.00 mg/m³).

^c The results derived from One-way ANOVA.

* $P < 0.05$, ** $P < 0.01$, got from Dunnett test comparing the control.

Table 4 Genetic polymorphism of metabolic enzymes in WBC counts as revealed by RFLP

Polymorphism	Number (%)	WBC $\pm$ SD ($\times 10^9$)	Beta	t	P
GSTM1					
Present	215 (55.8)	5.75 $\pm$ 1.67			
Null	170 (44.2)	5.43 $\pm$ 1.45	-0.11	-2.18	0.030
GSTT1					
Present	188 (48.8)	5.81 $\pm$ 1.68			
Null	197 (51.2)	5.42 $\pm$ 1.45	-0.10	-2.01	0.045
rs 1695					
AA	206 (53.5)	5.67 $\pm$ 1.60			
AG	158 (41.0)	5.58 $\pm$ 1.56	-0.04	-0.80	0.425
GG	21 (5.5)	5.24 $\pm$ 1.48	-0.06	-1.11	0.265
GG+AG	179 (46.5)	5.54 $\pm$ 1.55	-0.05	-1.03	0.305
rs3813867					
GG	262 (68.1)	5.74 $\pm$ 1.69			
GC	111 (28.8)	5.36 $\pm$ 1.30	-0.12	-2.45	0.014
CC	12 (3.1)	5.15 $\pm$ 1.25	-0.04	-0.83	0.410
CC+GC	123 (31.9)	5.34 $\pm$ 1.29	-0.13	-2.53	0.012
rs2031920					
CC	258 (67.0)	5.75 $\pm$ 1.63			
CT	113 (29.4)	5.35 $\pm$ 1.43	-0.12	-2.33	0.020
TT	14 (3.6)	5.04 $\pm$ 1.44	-0.07	-1.43	0.153
TT+CT	127 (33.0)	5.32 $\pm$ 1.43	-0.13	-2.56	0.011
rs6413432					
TT	242 (62.9)	5.62 $\pm$ 1.56			
TA	123 (31.9)	5.60 $\pm$ 1.64	-0.01	-0.14	0.893
AA	20 (5.2)	5.58 $\pm$ 1.55	0	0	1
TA+AA	143 (37.1)	5.61 $\pm$ 1.58	-0.01	0.12	0.90
rs1051740 ^a					
TT	92 (25.8)	5.54 $\pm$ 1.54			
TC	190 (53.2)	5.58 $\pm$ 1.58	0.04	0.66	0.508
CC	75 (21.0)	5.78 $\pm$ 1.63	0.08	1.29	0.197
TC/CC	265 (74.2)	5.63 $\pm$ 1.59	0.05	0.97	0.331
rs2234922					
AA	291 (75.6)	5.60 $\pm$ 1.49			
AG	90 (23.4)	5.64 $\pm$ 1.88	0.02	0.33	0.742
GG	4 (1.0)	5.35 $\pm$ 1.07	-0.01	-0.12	0.910
AG/GG	94 (24.4)	5.63 $\pm$ 1.85	0.02	0.30	0.774
mEH 3, 4					
Slow	263 (23.4)	5.64 $\pm$ 1.60			
Medium	83(1.0)	5.62 $\pm$ 1.56	-0.12	-0.60	0.547
Fast	11 (24.4)	4.85 $\pm$ 1.21	-0.70	-1.39	0.166

^a Data missing due to failure in DNA amplification.

Linear-regression analysis after adjusting gender, smoking, drinking, and age.

than the GSTM1-present group. The WBC in variant genotypes *CYP2E1* rs3813867 ($P=0.093$) and rs 2031920 ($P=0.070$) decreased more than the variant genotypes in the other three low CED levels.

Table 6 shows the association between WBC counts and polymorphisms of metabolism genes: *CYP2E1*, *mEH* and *GSTM1*, and *GSTT1* variants. The diplotype GCT/GCT and TA/TA that consists of the wild-type sequence for *CYP2E1* and *mEH* in all loci was selected as the reference. Among these diplotype pairs, the rare diplotypes (less than 1% frequency) were analyzed as a group. Compared with individuals with wild-type GCT/GCT and TA/TA, there was no significant difference compared to variant diplotypes.

The linear-regression analysis of the relationship between the genetic polymorphisms in metabolic enzyme genes and demographics factors on the decrease of WBC counts is found in Table 7. Age,

smoking, drinking, benzene exposure, *GSTM1*, and *GSTT1* were all associated with a reduced WBC count.

Discussion

The carcinogenic and hematotoxicity effects of benzene are well recognized by the scientific community. Significant decreases in white blood cell, red blood cell, and platelet counts have been observed in human populations exposed to relatively high levels of benzene. However, our understanding of toxicity related to low-level benzene exposure is limited, especially at levels below 1 ppm.²⁴ This study found evidence of low-level benzene concentrations having an effect on peripheral blood WBCs. This finding is consistent with other studies conducted in China among shoemakers,^{25–27} but contrary to a study in the Netherlands.²⁸ It is possible that the dose-relationship between low benzene exposure in exposed workers and the life exposure in internal controls was modified by other factors in the Netherlands study, such as small numbers, worker protection, and life factors (e.g. socio-demographic characteristics, eating habits, physical exercise).

Among smokers and older workers, WBCs declined significantly compared to non-smokers and younger workers, but was not significantly different when compare to the unexposed group. Female and older workers are more prone to be influenced by benzene than male and younger age groups. The apparent effect of smoking on WBC counts in PBL is somewhat puzzling. The most plausible interpretation for this is that the magnitude of association with benzene exposure was so strong that smoking relationships were masked. Alternatively, blood concentrations of the chemicals found in cigarettes might be too low to cause a WBC decrease in lymphocytes.

We found that total WBC counts significantly declined with increasing benzene CED and were lower in workers exposed to benzene at air levels of 1 ppm or less compared to unexposed. Lan *et al.*²⁵ reported that the decreases in white blood, red blood, and platelet counts were observed in workers exposed to relatively low levels of benzene (1 ppm). The findings were particularly robust because of the extensive exposure assessment over a 16-month period. However, the study in the Netherlands²⁸ showed no significant differences for any of the hematological parameters between the three exposure categories (<0.5, 0.5–1, and >1 ppm) or compared with the unexposed group. In our study, the WBC counts in the exposed group with high level of benzene-exposure (6.00 mg/m³) were higher than the exposed group with lower level of benzene-exposure (<3.25 mg/m³). Perhaps, the duration of

Table 5 The effect of cumulative exposure dose (CED) and metabolic enzymes genes in a multiplicative model among benzene-exposed workers

SNPs	5.02≤CED ^a ≤19.90			19.90<CED ^a ≤31.81			31.81<CED ^a ≤59.00			59.00<CED ^a ≤364.11		
	<i>n</i>	WBC±SD	<i>P</i> ^b	<i>n</i>	WBC±SD	<i>P</i> ^b	<i>n</i>	WBC±SD	<i>P</i> ^b	<i>n</i>	WBC±SD	<i>P</i> ^b
GSTM1												
Present	57	6.14±1.68		56	5.75±1.58		60	5.58±1.90		42	5.46±1.31	
Null	39	6.19±1.44	0.868	40	5.45±1.47	0.277	36	5.24±1.64	0.280	55	5.00±1.08	0.070
GSTT1												
Present	46	6.46±1.45		53	5.78±1.71		44	5.76±2.02		45	5.24±1.32	
Null	50	5.89±1.65	0.084	43	5.44±1.29	0.431	52	5.20±1.58	0.218	52	5.17±1.01	0.793
rs1695												
AA	49	6.51±1.68		58	5.59±1.42		48	5.34±1.83		52	5.28±1.19	
GG+AG	47	5.81±1.39	0.019	38	5.62±1.69	0.266	48	5.57±1.80	0.632	45	5.10±1.22	0.853
rs3813867												
GG	70	6.31±1.71		64	5.71±1.56		62	5.55±2.02		66	5.32±1.26	
CC+GC	26	5.78±1.09	0.122	32	5.46±1.49	0.620	34	5.28±1.35	0.398	31	4.93±1.04	0.093
rs2031920												
CC	73	6.22±1.56		65	5.70±1.52		55	5.71±2.09		65	5.32±1.24	
TT+CT	23	6.00±1.66	0.688	31	5.48±1.57	0.608	41	5.11±1.29	0.091	32	4.94±1.10	0.070
rs6413432												
TT	62	6.12±1.61		55	5.45±1.22		61	5.58±1.92		64	5.32±1.28	
TA+AA	34	6.26±1.53	0.888	41	5.87±1.86	0.179	35	5.24±1.61	0.386	33	4.96±	0.335
rs1051740												
TT	22	6.42±1.41		18	5.04±1.34		26	5.39±1.90		26	5.29±1.09	
TC+CC	65	6.04±1.64	0.311	69	5.78±1.55	0.038	66	5.57±1.79	0.155	66	5.57±1.79	0.923
rs2234922												
AA	77	5.99±1.41		71	5.61±1.49		75	5.49±1.77		68	5.29±1.12	
AG+GG	19	6.87±2.01	0.055	25	5.68±1.68	0.887	21	5.37±2.00	0.932	29	4.98±1.37	0.388

SNP, single nucleotide polymorphism.

^a The exposed workers were divided into four groups with quarter cumulative exposure dose (CED).^b *P* value derived from linear-regression analysis after adjusting gender, smoking, drinking, and age.

the exposure influenced the results, or the number of subjects in our lower WBC group was insufficient for stringent statistical comparison. It is possible that the benzene CED was more reflective of benzene-exposure than concentration.

Although the hematotoxicity and carcinogenicity of benzene have been reported for many decades, the mechanism by which genetic variations in metabolic

enzyme are prone to causes disease is not yet fully understood. It is speculated that genetic and lifestyle factors influence the toxic effects of benzene, but current evidence is inconclusive.^{29,30} Scientists postulate that the resultants of benzene metabolites are responsible for benzene toxicity because these metabolites can alkylate proteins and DNA.³¹ Previous studies have shown that individuals vary in their susceptibility to the adverse effects of benzene, probably due to differences in metabolic genes and corresponding enzymatic activity.^{12,32} It is postulated

Table 6 Associations between diplotypes of CYP2E1, mEH, and GSTM1/T1 and WBC counts

Diplotypes	<i>N</i> (%)	WBC (×10 ⁹)	Beta	<i>t</i>	<i>P</i>
CYP2E1 rs3813867, rs2031920, rs6413432					
GCT/GCT	182	5.67±1.59			
GCT/GCA	49	6.14±1.89	0.47	1.88	0.061
GCT/GTT	19	5.18±1.93	-0.39	-1.02	0.308
GCT/CCA	11	5.03±1.27	-0.69	-1.42	0.156
GCT/CTT	32	5.66±1.19	-0.03	-0.10	0.921
GCT/CTA	57	5.29±1.40	-0.36	-1.54	0.125
GCA/GCA	7	6.41±1.54	0.76	1.25	0.211
CTA/CTA	5	4.56±0.95	-0.84	-1.19	0.234
All others	23	5.42±1.32	-0.27	-0.79	0.430
mEH rs1051740, rs2234922					
TA/TA	81	5.63±1.56			
TA/TC	11	4.85±1.21	-0.28	-0.55	0.582
TA/CA	45	5.74±1.41	0.26	0.87	0.384
TA/CC	29	5.84±1.98	0.19	0.55	0.584
CA/CA	148	5.54±1.43	0.09	0.40	0.693
CA/CC	40	5.73±2.08	0.18	0.61	0.542
All others	31	5.61±1.57	0.17	0.53	0.598
GSTM1 GSTT1					
TM1+ TT1+	105	5.96±1.77			
TM1-TT1+	110	5.56±1.55	-0.43	-1.91	0.057
TM1+TT1-	83	5.63±1.58	-0.40	-1.91	0.057
TM1-TT1-	87	5.24±1.30	-0.66	-2.97	0.005

Table 7 Multiple linear analysis of WBC counts level of benzene-exposed workers

Variables	Beta	<i>t</i>	<i>P</i>
Gender ^a	-0.126	-1.935	0.054
Smoking ^b	0.145	2.415	0.016
Drinking ^c	-0.136	-2.154	0.032
Age ^d	-0.154	-3.130	0.002
CED ^e	-0.155	-3.162	0.002
GSTM1 ^f	-0.114	-2.322	0.021
GSTT1 ^f	-0.108	-2.202	0.028
rs 2031920 ^g	-0.098	-1.336	0.182
rs 3813867 ^h	-0.073	-1.005	0.316

^a Female vs male.^b Smoker vs non-smoker.^c Alcohol user vs alcohol non-user.^d The older group vs younger group.^e CED, cumulative exposed dose, which was continuous variable.^f Null vs non-null.^g TT+CT vs CC.^h CC+CG vs GG.

that when benzene-exposed workers cease occupational exposure, benzene metabolites in their bodies decrease gradually, with diminishing effects over time. If there is no significant damage in the function of DNA, the cellular functions should recover gradually, and the WBC will return to normal levels.

In this study, we assessed metabolizing enzyme gene polymorphisms that may affect individual susceptibility to hematotoxicity and tested eight polymorphism sites that were thought to affect enzymatic activities. It was recently reported that glutathione and GST appear to defend against benzene-induced DNA damage.³³ We found that benzene-exposed workers with *GSTT1* null genotypes and *GSTM1* null genotypes experienced lower WBC counts than others who did carry these genotypes. Previous research investigating metabolism gene polymorphisms and their association with chronic benzene poisoning reported that there was a 4.5-fold increased risk among workers carrying *GSTT1* null genotype (95% CI=1.13–17.54) compared with workers with *GSTT1* non-null genotype.³² Angelini *et al.* reported that the *GSTM1*-null genotype was associated with a significantly higher median micronucleus frequency in men. However, Bagryantseva *et al.* reported that the *GSTM1*-null genotype seemed to protect DNA integrity.^{14,15} A review reported that *GSTM1* and *GSTT1* showed some consistent associations with both biomarkers of exposure and effect.¹³ In line with expectations, we found an elevated WBC in benzene-exposed workers with *GSTT1* and *GSTM1*, respectively.

Microsomal epoxide hydrolase encodes an enzyme that initiates an alternate benzene oxide metabolic pathway, forming catechol. The polymorphisms mEH exon 3 (Tyr113His) and exon 4 (His139Arg) are associated with decreased and increased epoxide hydrolase activity, respectively. There was no statistically significant relationship between the mEH (rs1051740, rs2234922) and WBC counts. In an analysis comparing a group with predicted fast mEH activity to a group with slow mEH, the fast mEH group had lower WBC counts, although the difference was not statistically significant ($P=0.166$). This is consistent with previous research, and the variant alleles in rs2234922 polymorphisms are associated with aberrant promoter methylation of ERCC3 and reduced WBC counts in the benzene-exposed.³⁴ However, contrasting results were reported by Angelini *et al.*, with analysis revealing that high epoxide hydrolase (mEH) (predicted) enzyme activity was significantly correlated with a lower median MN frequency.¹⁴ Differences in the study design and samples (e.g. sample size, different plants, race, and exposure concentrations) may

explain the inconsistent results. The susceptibility of DNA repair genes was not considered.^{35,36}

Benzene is first activated by phase I enzymes, primarily cytochrome P450 2E1 (*CYP2E1*), into inter-mediate metabolites including PH, HQ, catechol, and 1,2,4-benzenetriol. These metabolites accumulate in bone marrow and undergo auto-oxidation or activation by peroxidases to yield the corresponding quinones, which are believed to be among the ultimate toxic metabolites of benzene. There is wide individual variation in the activity of this liver enzyme.³⁷ It has been reported that the overall effects of *RsaI* and *PstI* restriction polymorphism sites in the promoter region of the gene and in the *DraI* restriction site remain unclear.^{13,38} In this study, we found a weak, but statistically significant association, between the *CYP2E1* $-1293G>C$ (rs3813867), $-1019 C>T$ (rs2031920), and WBC counts. Individuals with variant alleles of rs3813867 and rs2031920 had lower WBC counts, although linear regression showed no apparent association between *CYP2E1* polymorphisms and WBC. This finding is consistent with previous studies,³⁹ reporting that rs3813867 *C/C* and rs2031920 *G/C* genotypes tended to be more susceptible to benzene toxicity. Carriers of the heterozygous variant might be associated with an increased risk of acute lymphoblastic leukemia (OR=2.8, 95% CI=1.2–6.7).³⁹ For rs2031920, homozygous variants produced fewer metabolites of PH, *E,E*-muconic acid (MA), and HQ than homozygous wild types.⁴⁰ Our study found similar results; the variant allele of *CYP2E1* in the promoter region (rs3813867, rs2031920) had lower levels of WBC counts. Perhaps, the variant alleles produced lower levels of benzene metabolites at a given benzene exposure than homozygous wild types, and that the effect was accentuated at higher benzene levels due to gene–environment interactions.

This finding may have an important implication for the prevention of chronic benzene poisoning in benzene-exposed workers. One merit of this study was a well-documented workplace exposure history to benzene. Besides, homogenous ethnic background of the subjects can enhance the persuasion of our conclusion, because confounders were theoretically reduced. There are also several limitations to our study. Worksite benzene concentrations were used to evaluate personal exposure level, instead of personal sampling. The robustness of our findings is limited because exposure assessment was measured three times in 1 day, not over a long time period. The joint effect between genetic polymorphisms and lifestyle factors on WBC counts is complicated, and small sample size does not have enough statistical power to detect gene–environment interactions. Additional

studies combining exposure data with WBC counts are warranted.

In summary, multiple linear analysis was used to detect the numerous effects of particular metabolizing genes and demographic and life styles on levels of peripheral blood WBC in 385 Chinese workers. We found that low-level benzene exposure (3.25 mg/m³ equal to 1 ppm) may lead to reduced WBC and individuals with null *GSTT1* and null *GSTM1* may contribute to the reduction of WBC from occupational exposure to benzene in our Chinese study population. Individuals carrying homozygotes for *CYP2E1* (rs3813867, *G>C*) and *CYP2E1* (rs2031920, *C>T*) polymorphism may have reduced WBC in peripheral blood relative to the homozygous wild-type subjects.

Disclaimer Statements

Contributors All the partner who took part in this manuscript have been enrolled as coauthors.

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References

- Infante PF. Benzene and leukemia, Pliofilm revisited: I. An historical review of the leukemia deaths among Akron Goodyear Tire and Rubber Company employees. *Int J Occup Environ Health*. 2013;19:215–22.
- Huff J. Benzene-induced cancers: abridged history and occupational health impact. *Int J Occup Environ Health*. 2007;13:213–21.
- Rinsky RA, Smith AB, Hornung R, Filloon TG, Young RJ, Okun AH, *et al.* Benzene and leukemia. An epidemiologic risk assessment. *N Engl J Med*. 1987;316:1044–50.
- Stockstad E. Toxicology. Factory study shows low levels of benzene reduce blood cell counts. *Science*. 2004;306:1665.
- Atkinson TJ. A review of the role of benzene metabolites and mechanisms in malignant transformation: summative evidence for a lack of research in nonmyelogenous cancer types. *Int J Hyg Environ Health*. 2009;212:1–10.
- Au WW, Ruchirawat M. Biomarkers in population studies: environmental mutagenesis and risk for cancer. *Rev Environ Health*. 2009;24:117–27.
- Lin YS, Vermeulen R, Tsai CH, Waidyanatha S, Lan Q, Rothman N, *et al.* Albumin adducts of electrophilic benzene metabolites in benzene-exposed and control workers. *Environ Health Perspect*. 2007;115:28–34.
- Ross D. The role of metabolism and specific metabolites in benzene-induced toxicity: evidence and issues. *J Toxicol Environ Health A*. 2000;61:357–72.
- Recio L, Bauer A, Faiola B. Use of genetically modified mouse models to assess pathways of benzene-induced bone marrow cytotoxicity and genotoxicity. *Chem Biol Interact*. 2005;15(3–154):159–64.
- Witz G, Zhang Z, Goldstein BD. Reactive ring-opened aldehyde metabolites in benzene hematotoxicity. *Environ Health Perspect*. 1996;104(Suppl 6):1195–9.
- Spatari G, Saitta S, Cimino F, Sapienza D, Quattrocchi P, Carrieri M, *et al.* Increased serum levels of advanced oxidation protein products and glycation end products in subjects exposed to low-dose benzene. *Int J Hyg Environ Health*. 2012;215:389–92.
- Sun P, Qian J, Zhang ZB, Wan JX, Wu F, Jin XP, *et al.* Polymorphisms in phase I and phase II metabolism genes and risk of chronic benzene poisoning in a Chinese occupational population. *Carcinogenesis*. 2008;29:2325–9.
- Dougherty D, Garte S, Barchowsky A, Zmuda J, Taioli E. NQO1, MPO, CYP2E1, GSTT1 and GSTM1 polymorphisms and biological effects of benzene exposure – a literature review. *Toxicol Lett*. 2008;182:7–17.
- Angelini S, Kumar R, Bermejo JL, Maffei F, Barbieri A, Graziosi F, *et al.* Exposure to low environmental levels of benzene: evaluation of micronucleus frequencies and S-phenylmercapturic acid excretion in relation to polymorphisms in genes encoding metabolic enzymes. *Mutat Res*. 2011;719:7–13.
- Bagryantseva Y, Novotna B, Rossner PJ, Chvatalova I, Milcova A, Svecova V, *et al.* Oxidative damage to biological macromolecules in Prague bus drivers and garagemen: impact of air pollution and genetic polymorphisms. *Toxicol Lett*. 2010;199:60–8.
- Ministry of Health of the People's Republic of China. Specifications of Air Sampling for Hazardous Substances Monitoring in the Workplace; 2004.
- Ministry of Health of the People's Republic of China. Determination of Toxic Substances in Workplace Air-Aromatic Hydrocarbons; 2007.
- Wang W, Qiu YL, Jiao J, Liu J, Ji F, Miao WB, *et al.* Genotoxicity in vinyl chloride-exposed workers and its implication for occupational exposure limit. *Am J Ind Med*. 2011;54:800–10.
- Wang Q, Wang AH, Tan HS, Feng NN, Ye YJ, Feng XQ, *et al.* Genetic polymorphisms of DNA repair genes and chromosomal damage in workers exposed to 1,3-butadiene. *Carcinogenesis*. 2010;31:858–63.
- Zhu SM, Ren XF, Wan JX, Xia ZL. Evaluation in vinyl chloride monomer-exposed workers and the relationship between liver lesions and gene polymorphisms of metabolic enzymes. *World J Gastroenterol*. 2005;11:5821–7.
- Yang B, O'Reilly DA, Demaine AG, Kingsnorth AN. Study of polymorphisms in the CYP2E1 gene in patients with alcoholic pancreatitis. *Alcohol*. 2001;23:91–7.
- Omer B, Ozbek U, Akkose A, Kilic G. Genetic polymorphism of cytochrome P450 2E1 in the Turkish population. *Cell Biochem Funct*. 2001;19:273–5.
- Wang Q, Tan HS, Zhang F, Sun Y, Feng NN, Zhou LF, *et al.* Polymorphisms in BER and NER pathway genes: effects on micronucleus frequencies among vinyl chloride-exposed workers in northern China. *Mutat Res*. 2013;754:7–14.
- Smith MT, Zhang L, McHale CM, Skibola CF, Rappaport SM. Benzene, the exposome and future investigations of leukemia etiology. *Chem Biol Interact*. 2011;192:155–9.
- Lan Q, Zhang L, Li G, Vermeulen R, Weinberg RS, Dosemeci M, *et al.* Hematotoxicity in workers exposed to low levels of benzene. *Science*. 2004;306:1774–6.
- Qu Q, Shore R, Li G, Jin X, Chen LC, Cohen B, *et al.* Hematological changes among Chinese workers with a broad range of benzene exposures. *Am J Ind Med*. 2002;42:275–85.
- McHale CM, Zhang L, Lan Q, Vermeulen R, Li G, Hubbard AE, *et al.* Global gene expression profiling of a population exposed to a range of benzene levels. *Environ Health Perspect*. 2011;119:628–34.
- Swaen GM, van Amelsvoort L, Twisk JJ, Verstraeten E, Slootweg R, Collins JJ, *et al.* Low level occupational benzene exposure and hematological parameters. *Chem Biol Interact*. 2010;184:94–100.
- McHale CM, Zhang L, Smith MT. Current understanding of the mechanism of benzene-induced leukemia in humans: implications for risk assessment. *Carcinogenesis*. 2012;33:240–52.
- Snyder R. Leukemia and benzene. *Int J Environ Res Public Health*. 2012;9:2875–93.
- Faiola B, Fuller ES, Wong VA, Recio L. Gene expression profile in bone marrow and hematopoietic stem cells in mice exposed to inhaled benzene. *Mutat Res*. 2004;549:195–212.
- Wan JX, Zhang ZB, Guan JR, Cao DZ, Ye R, Jin XP, *et al.* Genetic polymorphism of toxicant-metabolizing enzymes and prognosis of Chinese workers with chronic benzene poisoning. *Ann N Y Acad Sci*. 2006;1076:129–36.

- 33 Moro AM, Charao MF, Brucker N, Durgante J, Baierle M, Bubols G, *et al.* Genotoxicity and oxidative stress in gasoline station attendants. *Mutat Res.* 2013;754:63–70.
- 34 Xing C, Chen Q, Li G, Zhang L, Zheng M, Zou Z, *et al.* Microsomal epoxide hydrolase (EPHX1) polymorphisms are associated with aberrant promoter methylation of ERCC3 and hematotoxicity in benzene-exposed workers. *Environ Mol Mutagen.* 2013;54:397–405.
- 35 Xiao S, Gao L, Liu Y, Yu T, Jin C, Pan L, *et al.* Association of genetic polymorphisms in ERCC1 and ERCC2/XPD with risk of chronic benzene poisoning in a Chinese occupational population. *Mutat Res.* 2013;751:52–8.
- 36 Angelini S, Maffei F, Bermejo JL, Ravegnini G, L'Insalata D, Cantelli-Forti G, *et al.* Environmental exposure to benzene, micronucleus formation and polymorphisms in DNA-repair genes: a pilot study. *Mutat Res.* 2012;743:99–104.
- 37 Seaton MJ, Schlosser PM, Bond JA, Medinsky MA. Benzene metabolism by human liver microsomes in relation to cytochrome P450 2E1 activity. *Carcinogenesis.* 1994;15:1799–806.
- 38 Qu Q, Shore R, Li G, Su L, Jin X, Melikian AA, *et al.* Biomarkers of benzene: urinary metabolites in relation to individual genotype and personal exposure. *Chem Biol Interact.* 2005;153–154:85–95.
- 39 Wan J, Shi J, Hui L, Wu D, Jin X, Zhao N, *et al.* Association of genetic polymorphisms in CYP2E1, MPO, NQO1, GSTM1, and GSTT1 genes with benzene poisoning. *Environ Health Perspect.* 2002;110:1213–8.
- 40 Kim S, Lan Q, Waidyanatha S, Chanock S, Johnson BA, Vermeulen R, *et al.* Genetic polymorphisms and benzene metabolism in humans exposed to a wide range of air concentrations. *Pharmacogenet Genomics.* 2007;17:789–801.