

Association of Genetic Polymorphisms, mRNA Expression of *p53* and *p21* with Chronic Benzene Poisoning in a Chinese Occupational Population

Pin Sun,¹ Yulan Qiu,^{1,2} Zhongbin Zhang,¹ Junxiang Wan,¹ Tong Wang,³ Xipeng Jin,¹ Qing Lan,⁴ Nathaniel Rothman,⁴ and Zhao-lin Xia¹

¹Department of Occupational Health, School of Public Health, Fudan University, Shanghai, China, Key Laboratory of Public Health Safety (Fudan University), Ministry of Education, Shanghai, China; Departments of ²Health Toxicology and ³Health Statistics, School of Public Health, Shanxi Medical University, Taiyuan, China; and ⁴Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland

Abstract

DNA damage induced by benzene reactive metabolites is thought of as an important mechanism underlying benzene hematotoxicity and genotoxicity, and genetic variation in cell-cycle control genes may contribute to susceptibility to chronic benzene poisoning (CBP). Using a case-control study that included 307 benzene-poisoned patients and 299 workers occupationally exposed to benzene in south China, we aimed to investigate the association between genetic polymorphisms of *p53* and *p21* and the odds of CBP. To investigate whether benzene exposure may influence mRNA expression of *p53* and *p21* in benzene-exposed workers, we also chose 39 CBP workers, 38 occupationally benzene-exposure workers, and 37 nonexposure workers in the same region of China. PCR-restriction fragment length polymorphism technique was applied to detect polymorphisms of *p53* (rs17878362, rs1042522, and rs1625895) and

p21 (rs1801270 and rs1059234), and real-time PCR was applied to detect the quantity of gene mRNA expression. We found that *p21* C98A variant genotypes (CA+AA) or C70T variant genotypes (CT+TT) were associated with decreased odds of CBP [odds ratio (OR), 0.51; 95% confidence interval (95% CI), 0.32-0.83, and OR, 0.53; 95% CI, 0.29-0.95, respectively]. Further analysis showed the decreased odds of CBP in the subjects with *p21* CC/AT diplotype (OR, 0.51; 95% CI, 0.30-0.85). In addition, *p53* mRNA expression of CBP workers or benzene-exposure workers was significantly lower than that of nonexposure workers. Although these results require confirmation and extension, our results show that polymorphisms in *p21* may be protective against the risk of CBP in the Chinese occupational population. (Cancer Epidemiol Biomarkers Prev 2009;18(6):1821-8)

Introduction

Benzene, an important industrial chemical, is widely recognized as a prototypical hematotoxic and genotoxic carcinogen. Exposure to benzene can induce chronic benzene poisoning (CBP) characterized by hematotoxicities, leading to pancytopenia, aplastic anemia, and myelodysplastic syndrome (1, 2).

In spite of many years of research, the mechanism of how benzene causes those toxic and carcinogenic effects remains elusive. Earlier studies have shown that benzene reactive intermediates can bind covalently to macromolecules including DNA, tubulin, histones, and topoisomerase II in the tissue, and therefore cause genotoxicity as a result of oxidative damage to DNA or DNA double-strand breaks caused by reactive oxygen species (3, 4). Thus, DNA damage following benzene exposure must

be properly repaired to maintain genomic stability and to prevent proliferation of mutated cells and subsequent transformation into malignancies (5).

Besides DNA repair proteins, cell-cycle control proteins are also involved in DNA repair. P53, a well-known tumor suppressor protein, plays a central role in mediating cellular responses to DNA damage by acting as a transcription factor for genes involved in cell-cycle control, DNA repair, or apoptosis (6). In the presence of DNA damage after exposure to a carcinogen, P53 activated by specific phosphorylation events can increase expression levels of *p53*-responsive target genes, resulting in the arrest of the cell cycle in G₁ (7, 8) and preventing replication of damaged DNA. Previous studies have shown that loss of P53 function leads to genomic instability (9, 10), a common event in the development of human cancer (11). The *p53* gene is a known polymorphic, leading to variation in the change of gene expression and loss of this homeostatic control, inducing human carcinogenesis. So far, at least three *p53* polymorphisms have been reported to be involved in cancers: the 16-bp duplication in intron 3 (dbSNP rs17878362), a C-to-G transition at nucleotide 119 in exon 4 (C119G, codon 72, dbSNP rs1042522), and a G-to-A transversion at position 62 in intron 6 (IVS6+62 G>A, dbSNP rs1625895; ref. 12).

P21 (also known as Waf1/Cip1/CDKN1A) is one of the notable effectors of *p53* and is a general inhibitor of

Received 2/18/09; revised 3/20/09; accepted 4/2/09; published online 6/8/09.

Grant support: National Natural Science Foundation of China grant (grant number 30271113), China National Key Basic Research and Development Program grant (grant number 2002CB512902), Scientific and Technical Supporting Programs of China grant (grant number 2006BAK05B01), and grants from Shanghai Bureau of Public Health (grant number 08GWD12 and 08GWZX0402).

Requests for reprints: Zhao-lin Xia, Department of Occupational Health, School of Public Health, Fudan University, Shanghai, China; Key Laboratory of Public Health Safety (Fudan University), Ministry of Education, Shanghai, China. Phone: 86-21-54237050; Fax: 86-21-54237050. E-mail: zlxia@shmu.edu.cn

Copyright © 2009 American Association for Cancer Research.

doi:10.1158/1055-9965.EPI-09-0140

Table 1. Characteristics of selected demographic and exposure variables in CBP cases and controls

	CBP cases	Healthy controls	P*
	No. (%)	No. (%)	
Total	307 (100.0)	299 (100.0)	
Age (y)			0.500
≤25	85 (27.7)	86 (28.8)	
26-35	117 (38.1)	97 (32.4)	
36-45	81 (26.4)	88 (29.4)	
>45	24 (7.8)	28 (9.4)	
Gender			0.870
Male	195 (63.5)	188 (62.9)	
Female	112 (36.5)	111 (37.1)	
Exposure duration (y)			0.252
≤5	150 (48.9)	160 (53.5)	
6-10	62 (20.2)	66 (22.1)	
11-15	37 (12.1)	35 (11.7)	
16-20	25 (8.1)	20 (6.7)	
>20	33 (10.7)	18 (6.0)	
Intensity of exposure (mg/m ³)			0.687
≤40	187 (60.9)	190 (63.5)	
40-100	91 (29.6)	86 (28.8)	
>100	29 (9.5)	23 (7.7)	
Smoking			0.046
Yes	154 (50.2)	137 (45.8)	
No	30 (9.8)	45 (15.1)	
No data	123 (40.0)	117 (39.1)	
Alcohol consumption			0.404
Yes	148 (48.2)	151 (50.5)	
No	38 (12.4)	31 (10.4)	
No data	121 (39.4)	117 (39.1)	

* χ^2 test for difference in the distributions between the two groups.

cyclin-dependent kinases. It functions to negatively control the cell cycle (13, 14). The expression of *p21* is up-regulated by *p53* in response to DNA damage, leading to cell-cycle arrest at the G₁ checkpoint (14). *In vitro* studies have shown that *p21* expression can suppress tumor growth (15), and reduced *p21* expression in tumor has been associated with poor prognosis in humans (16, 17). Thus, genetic variants in *p21* may modulate its expression and thereby affect carcinogenesis. Among the single nucleotide polymorphisms (SNP) of *p21*, two SNPs were reported to be associated with cancers (18, 19): one is at nucleotide 98 in exon 2, which causes a nonsynonymous serine-to-arginine substitution at codon 31(C98A, dbSNP rs1801270), and the other locates the 3' untranslated region and causes a single C-to-T substitution at nucleotide 70 in exon 3 (T70C, dbSNP rs1059234).

Because exposure to benzene can induce DNA damage and therefore activate the *p53*-regulated biological defense responses, we hypothesized that polymorphisms of *p53* and *p21* may contribute to the risk of CBP. In addition, *in vitro* studies indicated that inhaled benzene can influence the expression of the cell-cycle control genes including *p53* and *p21* (5, 20), but it is yet unclear that the expression of *p53* and *p21* in peripheral blood cells would be affected by exposure to benzene. In this study, therefore, we conducted a case-control study to investigate the effects of SNPs in *p53* and *p21* on human susceptibility to CBP and the alteration in mRNA level of *p53* and *p21* among CBP workers, benzene-exposed workers, and non-exposed workers in a Chinese occupational population.

Materials and Methods

Subjects. The details of this case-controls study have been described elsewhere (2, 21-23). Briefly, 267 of 307

CBP patients in this study came from six factories in Shanghai, Hangzhou, Maanshan, Wuhu, Cixi, and Guangzhou, where clusters of cases were reported. Another 40 patients, who returned to the hospital periodically for health examinations, were from 12 other small factories that had been closed down. The benzene poisoning was diagnosed from 1980 to 2005 by the local authorized Occupational Disease Diagnostic Team. The diagnostic criteria for occupational benzene poisoning, according to the Ministry of Health of China, have also been described previously (2, 21). A total of 299 healthy workers who had been occupationally exposed to benzene in the six major factories were selected as controls. Control subjects were frequency-matched to cases by gender, age within 5 y, employment duration of the same working environment within 3 y, and exposure level at the workplace. The calendar range for the start of employment of the cases and the controls was between 1958 and 2005 and between 1963 and 2005, respectively. To explore the effect of benzene on gene expression of *p53* and *p21*, we chose 39 workers with CBP as cases (22 male and 17 female; mean age, 34.1 y) and 38 healthy workers occupationally exposed to benzene as benzene-exposed controls (22 male and 16 female; mean age, 33.9 y) from the same factory. We also chose 37 workers who were not exposed to benzene as unexposed controls (13 male and 24 female; mean age, 45.3 y) in the same region of China.

Prior to the study, written informed consent was obtained from each subject and a questionnaire was used to determine the lifestyle of each subject, namely ethnic background, nutrition, cigarette smoking and alcohol consumption, protective measures, self-reported symptoms, medical history, and occupational history such as work unit (department), type of work, and exposure duration. Exposure estimation was based on monitoring data or industrial hygienists and long-term employees' evaluation considering historical changes (24). The intensity of benzene exposure (milligrams per cubic meter) for the patients was taken as the benzene level of workplaces while diagnoses were made; the intensity of benzene exposure for the controls was taken as the current level monitored by organic vapor passive dosimetry badges during collection of the blood samples. Those who smoked at least 1 cigarette per day for >1 y were considered regular smokers. Alcohol intake was defined as drinking at least 7 standard units of alcohol on average per week [1 standard unit = 10 g of alcohol equivalent; e.g., a glass/can/bottle (330 mL) of regular beer (5%), a measure (40 mL) of liquor, a glass (120 mL) of wine] for >6 mo. The subjects were administered a rigorous physical examination at a local occupational disease hospital. Alanine aminotransferase level in serum was also examined for liver function evaluation.

Genotyping Assay. Genomic DNA was isolated from peripheral blood leukocytes using a standard method and stored at -80°C until analysis. *p53* and *p21* genotypes were determined using PCR and restriction fragment length polymorphism assays previously described (25, 26). The genotyping data were recorded by two independent readers who were blinded to the case-control status of the samples. At least of 10% of DNA randomly selected samples were run in duplicates to ensure quality control, and the results were 100% concordant.

Table 2. Genotypes for genetic polymorphisms in *p53* and *p21* and their effects on risk of CBP

Genotype	Cases (%) [*]	Controls (%) [*]	OR (95% CI)	OR _{adj.} (95% CI) [†]
<i>P53</i> intron 3 [‡]				
WW	280 (94.6)	258 (92.8)	1.00	1.00
WM	16 (5.4)	20 (7.2)	0.74 (0.37-1.45)	% (0.22-1.58)
MM	0 (0.00)	0 (0.0)	—	—
WM+MM	16 (5.4)	20 (7.2)	0.74 (0.37-1.47)	% (0.22-1.58)
<i>P53</i> C119G				
CC	56 (18.7)	51 (17.8)	1.00	1.00
CG	144 (48.0)	142 (49.5)	0.92 (0.59-1.44)	1.04 (0.58-1.87)
GG	100 (33.3)	94 (32.8)	0.97 (0.60-1.55)	0.87 (0.46-1.63)
CG+GG	244 (81.3)	236 (82.2)	0.94 (0.62-1.43)	0.97 (0.55-1.69)
<i>P53</i> IVS6+62 G>A				
GG	272 (89.8)	265 (90.1)	1.00	1.00
GA	31 (10.2)	29 (9.9)	1.04 (0.61-1.78)	1.28 (0.63-2.57)
AA	0 (0.00)	0 (0.0)	—	—
GA+AA	31 (10.2)	29 (9.9)	1.04 (0.61-1.78)	1.28 (0.63-2.57)
<i>P21</i> C98A				
CC	100 (33.0)	65 (22.5)	1.00	1.00
CA	158 (52.2)	173 (59.9)	0.59 (0.41-0.87) [§]	0.48 (0.29-0.79) [§]
AA	45 (14.9)	51 (17.6)	0.57 (0.35-0.95)	0.67 (0.33-1.35) [§]
CA+AA	203 (67.0)	224 (77.5)	0.59 (0.41-0.85) [§]	0.51 (0.32-0.83) [§]
<i>P21</i> C70T				
CC	66 (21.9)	45 (15.4)	1.00	1.00
CT	104 (34.6)	107 (36.6)	0.66 (0.42-1.06)	0.56 (0.29-1.06)
TT	131 (43.5)	140 (48.0)	0.64 (0.41-1.00)	0.51 (0.28-0.94)
CT+TT	235 (78.1)	247 (84.6)	0.65 (0.43-0.99)	0.53 (0.29-0.95)

*Data missing due to failure in DNA amplification.

[†]ORs were adjusted for potential confounders including gender, cigarette smoking, alcohol consumption, exposure duration, and intensity of benzene exposure.

[‡]WW, wild-type homozygous; WM, wild-type/mutant heterozygous; MM, mutant homozygous.

[§] $P < 0.01$.

^{||} $P < 0.05$.

Total RNA Extraction and Reverse Transcription-PCR and Real Time PCR. Lymphocytes were isolated from 2 mL anticoagulated peripheral blood using a standard method. Trizol (Gibco BRL Products) was added to the cells and was agitated between three and five times. The mixture was then stored at -80°C until use.

Total RNA was isolated from the lymphocytes and quantified by measurement of the absorbance at 260 nm; purity was assessed from the 260/280 nm absorbance ratio. Reverse transcription-PCR was done using the one-step SuperScript kit (Gibco BRL) according to the manufacturer's instructions. A total reaction mixture volume of 25 μL contained 3 μg of RNA.

The quantity of *p53* and *p21* gene mRNA expression was detected by real-time PCR (SYBR Green I) with PRISM ABI 7900HT Sequence Detection System (Applied Biosystem). Expression of the housekeeping gene glyceraldehyde 3-phosphoric acid dehydrogenase (*GAPDH*)

was used for normalization of *p53* and *p21* mRNA to enable cross-comparisons among the samples. All the primers were designed by using the Primer premier software 5.0 (PREMIER Biosoft International). Specificity of primers was confirmed by homology search. The *p53* primers were 5'-TGACTGTACCACCATCCACTA-3' (sense) and 5'-AAACACGCACCTCAAAGC-3' (antisense; GI:20407067), giving rise to a 146 bp PCR production. The *p21* primers were 5'-TGGACCTGTCACTGTCTTGT-3' (sense) and 5'-TCCTGTGGGCGGAT-TAG-3' (antisense; GI: 7978496), giving rise to a 176 bp PCR production. The *GAPDH* primers were 5'-AGGGCTGCTTTTAACTCTG-3' (sense) and 5'-CTGGAAGATGGTGATGGG-3' (antisense; GI: 3641890), giving rise to a 177 bp PCR production. The real-time PCR was done using 50 ng of cDNA, 0.25 μL of each

Table 3. Haplotypes/diplotypes of *p53* and the odds of chronic benzene poisoning

Haplotype/Diplotypes [*]	Cases (%) [†]	Controls (%) [†]	OR (95% CI)	χ^2	<i>P</i>
Haplotypes					
WCC	233 (38.1)	222 (37.6)	1.00		
WGC	345 (56.4)	335 (56.8)	0.98 (0.77-1.24)	0.02	0.876
MCA	13 (2.1)	16 (2.7)	0.77 (0.36-1.65)	0.44	0.505
WCA	14 (2.3)	12 (2.0)	1.11 (0.50-2.46)	0.07	0.794
Diplotypes					
WCC/WCC	45 (15.1)	44 (15.2)	1.00		
WCC/WGC	132 (44.3)	126 (43.5)	1.02 (0.63-1.66)	0.01	0.922
WGC/WGC	95 (31.9)	92 (31.7)	1.01 (0.61-1.67)	0.00	0.970
Other diplotypes [‡]	26 (8.7)	28 (9.6)	0.91 (0.46-1.79)	0.08	0.780

*The allele order is intron3, C119G, and IVS6+62 C>A from left to right.

[†]Data missing due to failure in DNA amplification.

[‡]All subgroups with frequencies <5% were combined as one group.

Table 4. Haplotypes/diplotypes of p21 and the odds of chronic benzene poisoning

Haplotype/Diplotypes*	Cases (%) [†]	Controls (%) [†]	OR (95% CI)	χ^2	P
Haplotype					
CC	224 (37.2)	184 (31.5)			
AT	235 (39.0)	267 (45.7)			
AC	131 (21.8)	120 (20.6)			
CT	12 (2.0)	13 (2.2)			
Diplotypes					
CC/CC	60 (19.9)	37 (12.7)	1.00		
CC/AT	75 (24.9)	91 (31.2)	0.51 (0.30-0.85)	6.81	0.009
CC/CT	26 (8.6)	15 (5.1)	1.07 (0.50-2.28)	0.30	0.863
AT/AT	39 (13.0)	48 (16.4)	0.50 (0.28-0.90)	5.35	0.021
AT/CT	79 (26.3)	79 (27.1)	0.62 (0.37-1.03)	3.41	0.065
Other diplotypes [‡]	22 (7.3)	22 (7.5)	0.62 (0.30-1.27)	1.75	0.186

*The allele order is C98A and C70T from left to right.

[†]Some data were missing due to inability to amplify DNA.

[‡]All subgroups with frequencies <5% were combined as one group.

primer (10 μ mol/L), and 2 \times ABsoluteTM QPCR SYBR Green Mix (including 500 nmol/L ROX, ABgene) in a 10 μ L reaction volume. PCR program was a 15-min activation step at 95°C, followed by 40 cycles of 95°C for 15 s, and finally 60°C for 1 min. Every sample was done by three parallels. The results were analyzed by PRISM ABI 7900HT Sequence Detection System (Applied Biosystem). The threshold cycle (C_t) values for the triplicate reactions were averaged and the average *GAPDH* C_t value for each sample was subtracted from the average C_t value of interest to obtain a normalized C_t value. The mRNA expression of objective gene was shown by $2^{-\Delta C_t}$ value. To simplify the presentation of the data, the relative expression values were multiplied by 10^3 .

Statistical Analysis. All analyses were done using SPSS 10.0 software (SPSS Inc.). Each SNP was tested in controls to ensure fitting with Hardy-Weinberg equilibrium. Frequencies of the different genetic polymorphisms were compared between cases and controls by Fisher's exact test or χ^2 test, when appropriate. To evaluate whether the lifestyle factor modified the association between genetic polymorphisms and odds of CBP, χ^2 test was applied after stratification according to cigarette smoking or alcohol consumption. The test for homogeneity of odds ratios (OR) was examined by the Breslow-Day method. The OR and 95% confidence interval (95% CI) for estimating the associations of genetic polymorphisms with the odds of CBP were obtained from unconditional logistic regression models without and with adjustment for potential confounders including gender, cigarette smoking, alcohol consumption, exposure duration, and intensity of benzene exposure. Linkage disequilibrium analysis was tested by the method described by Shi and He (27). Haplotype and diplotype frequencies were estimated using a Bayesian approach implemented with PHASE (28, 29). The frequency distribution of the haplotypes and diplotypes was calculated by χ^2 analysis. Two-tailed $P < 0.05$ was considered statistically significant. To account for multiple testing, we applied the Bonferroni correction.

Due to the nonnormal distribution of gene expression data, values of *p53* and *p21* mRNA expression were reported as median and interquartile range. In order to adjust for the influence of demographic characteristics such as gender, cigarette smoking, and alcohol consumption, we did a multiple factor analysis using robust multiple regression, with PROC ROBUSTREG under SAS Version

9.1 (SAS Institute Inc.). Two-tailed $P < 0.05$ was considered statistically significant.

Results

Demographics of Cases and Controls. The distributions of age, gender, exposure duration, cigarette smoking, and alcohol consumption in the cases and controls are shown in Table 1. The median age and exposure duration was 31 years (range, 18-57) and 4.5 years (range, 1-38) in 307 CBP cases, respectively, and 32 years (range, 18-68) and 4 years (range, 1-36), in 299 controls, respectively. There was no significant difference in the distribution of age (≤ 25 , 26-35, 36-45, >45 years), exposure duration (≤ 5 , 6-10, 11-15, 16-20, >20 years), intensity of benzene exposure (≤ 40 mg/m³, 40-100 mg/m³, >100 mg/m³), and gender ($P > 0.05$ for all), suggesting that frequency-matching was adequate. Because the data were in part coming from historical records, some records were incomplete for data on cigarette smoking or alcohol consumption. For those who had historical data, the percentage of females was 60.0% in the cases and 60.9% in the controls for those who had cigarette smoking data, and 60.6% in cases and 60.9% in controls who had alcohol consumption data. The range for total WBC counts in the controls was from 4,500/ μ L to 5,900/ μ L; the mean was $4,838 \pm 259$ / μ L.

Genetic Polymorphisms of p53 and p21. The frequency distributions of genotypes of *p53* and *p21* in this study population revealed that no mutant homozygous (MM) genotype of *p53* intron 3 and AA genotype of *p53* IVS6 +62 G>A were detected (Table 2). The variant genotype frequency of *p21* C98A polymorphism was significantly different between cases and controls ($P < 0.05$), but the

Table 5. Demographic characteristics of the study subjects

Variables	Nonexposed	Benzene-exposed	CBP
Number	37	38	39
Age (y)	45.4 \pm 5.9*	33.0 \pm 7.3	33.4 \pm 7.8
Male (%)	15 (34.9)	22 (57.9)	22 (56.4)
Current smoker (%)	14 (32.6)	14 (36.8)	12 (30.8)
Current alcohol user (%)	12 (27.9)	14 (36.8)	19 (48.7)

* $P < 0.05$.

Table 6. *p53* and *p21* mRNA expression ($2^{-\Delta Ct} \times 10^3$) in the study subjects

Gene	Nonexposed (<i>n</i> = 37)	Benzene-exposed (<i>n</i> = 38)	CBP (<i>n</i> = 39)
<i>p53</i>	16.40 (9.62, 26.83)	2.16 (0.92, 4.03)*	1.61 (0.33, 4.07)*
<i>p21</i>	4.02 (2.39, 5.05)	4.05 (1.79, 7.49)	3.90 (1.32, 8.52)

* $P < 0.001$ (robust multiple regression analysis).

other variant genotypes frequencies were not ($P > 0.05$ for all). The distributions of the genotypes of these genetic polymorphisms in the controls were in Hardy-Weinberg equilibrium ($P > 0.05$) except the genotypes of *p21* C98A and *p21* C70T.

Effect of Genetic Polymorphism in *p53* and *p21* on the Risk of CBP. For the *p53* gene, no statistical difference was found in the distribution of genotypes of *p53* intron 3, C119G, and IVS6+62 G>A ($P > 0.05$) in this population. However, the proportion of individuals carrying *p21* 98 CA+AA, or *p21* 70 CT+TT was lower in cases (67.0% and 78.1%, respectively) than in controls (77.5% and 84.6%, respectively). Correspondingly, the odds of CBP for individuals carrying *p21* 98 CA+AA genotypes or *p21* 70 CT+TT genotypes was 0.51-fold lower (OR_{adj}, 0.51; 95% CI, 0.32-0.83; $P = 0.006$) or 0.53-fold lower (OR_{adj}, 0.53; 95% CI, 0.29-0.95; $P = 0.032$) compared with those carrying the *p21* 98 CC genotype or the *p21* 70 CC after adjustment for potential confounders such as gender, cigarette smoking, alcohol consumption, exposure duration, and intensity of benzene exposure.

Haplotypes of Polymorphisms in *p53* and *p21* on the Risk of CBP. To examine the linkage among the three loci (intron 3, exon 4, and intron 6) of *p53*, we did linkage disequilibrium analyses. The D' values were 0.831 (intron 3 with exon 4), 0.796 (intron 3 with intron 6), and 0.599 (exon 4 with intron 6). We also examined the linkage between the two loci of *p21*, and the D' value was 0.834.

Based on the observed genotypes of *p53* and *p21* in this study population, we reconstructed their haplotypes and diplotypes (Tables 3 and 4). For *p53*, four haplotypes were reconstructed applied by the Phase software. However, no significant differences were observed in the distribution of their haplotypes in this population, and further analysis showed that there were no significant differences in the distribution of their diplotypes either (Table 3). For *p21*, four haplotypes were found in this population. Because the polymorphisms of *p21* in this present study departed from Hardy-Weinberg disequilibrium, we only investigated the role of *p21* diplotypes on the odds of CBP. The results showed that there were statistically significant differences in the distributions of those diplotypes in cases and those in controls. Using the Bonferroni correction with five tests (requiring $P < 0.01$), a statistically significant difference was observed in the distribution of CC/AT diplotypes ($P = 0.009$; Table 4). Compared with those carrying the CC/CC diplotypes, subjects with CC/AT diplotype had a decreased odds of CBP (OR, 0.51; 95% CI, 0.30-0.85).

Demographic Characteristics of the Study Subjects for the Study of mRNA Expression. The three groups matched on the basis of gender as well as smoking and drinking habits, but the CBP and benzene-exposed

groups were significantly younger than the unexposed group ($P < 0.05$; Table 5).

mRNA Expression of *p53* and *p21*. Robust multiple regression indicated that *p53* mRNA expression of cases or benzene-exposure controls was significantly lower than that of the nonexposure controls ($P < 0.001$), but the difference of *p21* mRNA expression between these three groups was not significant ($P = 0.695$; Table 6).

Discussion

In the present study, we studied the effects of polymorphisms in the *p53* and *p21* genes on the risk of CBP among 307 CBP workers and 299 benzene-exposed workers in China. We also investigated the effect of benzene exposure on the *p53* and *p21* mRNA expression in the peripheral blood lymphocytes among 39 CBP workers, 38 benzene-exposed controls, and 43 nonexposure controls. Although no significant association was found among three polymorphisms (intron 3, C119G, and IVS6+62 G>A) in *p53* and the odds of CBP, our results showed that benzene exposure decreased the mRNA expression of *p53* in human peripheral blood. In contrast to the *p53* gene, our results showed that two SNPs in *p21* (C98A and C70T) were associated with the decreased odds of CBP, but no significant differences in the *p21* gene mRNA expression were observed among CBP workers, benzene-exposed controls, and nonexposure controls.

In response to a variety of stress signals, including genotoxic stress, *p53* is activated, leading to the initiation of a range of biological defense pathways. Through *p53*-mediated cell-cycle arrest, cells are allowed sufficient time to repair DNA damage and replication errors. Different studies suggest that *p53* seems to play a key role in benzene-induced hematotoxicity, and its dysfunction is a potential factor in benzene-induced carcinogenesis (30, 31).

Altering the amino-acid sequence of its product, the C119G/R72P polymorphism in *p53* can affect its biochemical properties and biological functions (12). Several epidemiologic studies have found that this polymorphism was associated with the risk of different types of cancer, but the results are conflicting (32-34). Recently, Shen et al. (35) reported that the *p53* C119G polymorphism was associated with a statistically significant decrease in total WBC counts among exposed workers. In the current study, however, we failed to observe an association between this SNP and the susceptibility to CBP.

In addition, we investigated two polymorphisms in *p53* noncoding regions. Among all of the polymorphisms identified in *p53*, the most frequently studied have been the polymorphism in intron 3 (16 bp duplication) and the codon 72 polymorphism. Some investigators reported that the +16 allele (mutant allele) was associated with decreased apoptotic and DNA repair capacity in

lymphoblastoid cell lines (25). The other *p53* polymorphism we studied was a G-to-A transversion at position 62 in intron 6. This SNP was found to be associated with altered *p53* function (36). Epidemiologic studies have correlated the intron 3 duplication or the intron 6 polymorphism to increased risk of various cancers (25, 37-39). The present study, by contrast, failed to observe the effect of these two polymorphisms of *p53* on the susceptibility to CBP.

In order to confirm the effects of *p53* polymorphisms on individual susceptibility to CBP, we applied the Phase software to reconstruct its haplotypes and diplotypes. Our results showed no association between *p53* haplotypes or diplotypes and the odds of CBP. There are some plausible explanations for our findings. First, the variant genotypes of intron 3 or intron 6 are uncommon in Chinese populations. The low prevalence of these two genotypes would make it difficult to detect their association with the odds of CBP in our study. Second, *p53* polymorphisms combined with either environmental factors such as smoking (40), or certain conditions such as disease types, disease stages, and age of onset (41-43), are likely to contribute to the risk of cancers. Women seldom smoked cigarettes. Yet in this study, most of those who had cigarette smoking were female, potentially introducing bias in our results.

In the present study, we also detected *p53* mRNA expression in peripheral blood lymphocytes between benzene-exposed workers and nonexposure workers. Earlier *in vitro* studies (30, 31) showed that *p53* was a central regulator of the bone marrow response to inhaled benzene. Our finding that there was a significant difference in the mRNA expression among CBP workers, benzene-exposed controls, and nonexposure controls, confirmed prior findings in those studies. Our result indicated that by decreasing the *p53* mRNA expression in peripheral blood lymphocytes, exposed benzene might affect cell-cycle arrest, leading to CBP and leukemia. But this mechanism is unclear. Considering the bone marrow is the main target tissue of benzene, further study is needed to confirm this hypothesis.

Two previously reported SNPs in *p21* were found in the subjects enrolled in our study. Between the two SNPs, the codon 31 polymorphism (C98A) is the only one located in the coding region. Su et al. (44) reported that this polymorphism may alter mRNA expression of *p21*. Interestingly, the other polymorphism of *p21* tested in our study (*p21* C70T) is linked to the C98A polymorphism (12). The C70T polymorphism is located in the 3' untranslated region, and in turn, the 3' untranslated region is a region that controls mRNA stability and degradation. It has been suggested that the C70T polymorphism may prevent timely degradation of the *p21* mRNA, therefore influencing the cellular DNA damage-induced cell cycle arrest response (26, 45). Although a number of published studies have examined the effect of *p21* polymorphisms on human cancers, as far as we know, this is the first study to explore the role of *p21* polymorphisms in the susceptibility to CBP. In the present study, we observed that after adjustment for potential confounders, individuals carrying *p21* 98A variant genotypes or *p21* 70T variant genotypes decreased the odds of CBP. Further analysis indicated that workers carrying *p21* CC/AT diplotype are less susceptible to CBP, a finding consistent with a number of studies in which these two SNPs of *p21* correlate

with cancer risk (26, 45-48). Therefore, these two SNPs may play a role in susceptibility to CBP.

Although P53 does not actively repair DNA, it functions to activate *p21* to regulate cell-cycle checkpoints that can pause the cell cycle to allow for DNA repair. Therefore, it is expected that *p21* plays an important role in the *p53*-mediated cell cycle arrest in response to DNA damage. In contrast to *p53*, we failed to find that benzene exposure altered the *p21* mRNA expression in the peripheral blood lymphocytes. Faiola et al. (5) found that in mice, *p21* mRNA was not altered in hematopoietic stem cells following benzene exposure. This may explain, in part, why no apparent difference of *p21* mRNA expression in the peripheral blood lymphocytes among the three groups was found in this study.

Although genetic polymorphisms play an important role in the expression of diseases, most chronic and complex illnesses are likely caused by interactions among environmental exposure, genetic polymorphisms, and lifestyle behaviors (21), such as tobacco and alcohol consumption, all of which are known risk factors for CBP (49, 50). This study found reduced odds of CBP for non-alcohol users with the *p21* 98A or 70T allele and nonsmokers with the *p21* 98A allele. However, the test for homogeneity of odds ratios suggested no potential interactions between those polymorphisms and tobacco/alcohol consumption. In addition, the relative low frequency of alcohol and tobacco consumption may have introduced some bias in our results; the majority of subjects with relevant information available were females who also were less likely than males to have indulged in alcohol and cigarette use. As such, replication of our findings in other benzene-exposed populations is critical.

Furthermore, accurate exposure estimation is quite important for evaluating exposure levels between the case and control groups. Because not all cases and controls are in the same exposure environment due to different types of work, we were limited to evaluating the intensity of benzene exposure according to methods described by Dosemeci et al. (24). That is, exposure levels were measured differently between cases and controls; it may not completely reflect individual variation in exposure. To minimize the potential for bias, more accurate methods for exposure estimation must be introduced, including individual sampling, which is more accurate than the traditional area sampling used in this study. Benzene metabolites reflecting the exposure levels also are needed to be applied in accurate exposure estimation.

We found for the first time that genetic variants in *p21* may contribute to the development of CBP in occupational exposure to benzene in a Chinese population. This finding may have important implications for the prevention of CBP in susceptible workers. Although this study included a homogeneous ethnic background of the subjects, well-documented workplace exposure history to benzene, and a frequency-matching design, it is possible that some associations will be false-positives, particularly those based on small numbers of subjects carrying at-risk alleles after adjustment for potential confounders. In addition, joint action between genetic polymorphisms and lifestyle risk factors on special diseases such as CBP is complicated, and small studies like the present one do not have enough statistical power to detect gene-environment interactions.

Thus, a more comprehensive, larger-scale study is needed to further explore the effects of gene-environment interaction on genetic susceptibility to CBP.

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.

We thank Duozhi Cao (Institute of Occupational Health attached to Maanshan Steel & Iron Group, Maanshan, Anhui, China), Rong Ye and Jiru Guan (Hangzhou Center for Diseases Control and Prevention, Hangzhou, Zhejiang, China), Weiwei Liu (Guangzhou Hospital for Occupational Diseases, Guangzhou, Guangdong, China), and Yuejun Zheng (Cixi Center for Diseases Control and Prevention, Cixi, Zhejiang, China), who are physicians, for their contribution to field research and physical examination in this study; and Dr. Qingyi Wei (The University of Texas M. D. Anderson Cancer Center) for his critical review and scientific editing.

References

- Capleton AC, Levy LS. An overview of occupational benzene exposures and occupational exposure limits in Europe and North America. *Chem Biol Interact* 2005;153:43–53.
- Zhang Z, Wan J, Jin X, et al. Genetic polymorphisms in XRCC1, APE1, ADPRT, XRCC2, and XRCC3 and risk of chronic benzene poisoning in a Chinese occupational population. *Cancer Epidemiol Biomarkers Prev* 2005;14:2614–9.
- Andreoli C, Leopardi P, Crebelli R. Detection of DNA damage in human lymphocytes by alkaline single cell gel electrophoresis after exposure to benzene or benzene metabolites. *Mutat Res* 1997;377:95–104.
- Amin RP, Witz G. DNA-protein crosslink and DNA strand break formation in HL-60 cells treated with trans,trans-muconaldehyde, hydroquinone and their mixtures. *Int J Toxicol* 2001;20:69–80.
- 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.
- Vousden KH. Outcomes of p53 activation-spoilt for choice. *J Cell Sci* 2006;119:5015–20.
- el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817–25.
- Venkatachalam S, Shi YP, Jones SN, et al. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J* 1998;17:4657–67.
- Harvey M, Sands AT, Weiss RS, et al. *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* 1993;8:2457–67.
- Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* 1992;70:923–35.
- Hainaut P, Hernandez T, Robinson A, et al. IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. *Nucleic Acids Res* 1998;26:205–13.
- Pietsch EC, Humbey O, Murphy ME. Polymorphisms in the p53 pathway. *Oncogene* 2006;25:1602–11.
- Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993;75:805–16.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993;366:701–4.
- Moffatt KA, Johannes WU, Hedlund TE, Miller GJ. Growth inhibitory effects of 1 α , 25-dihydroxyvitamin D(3) are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. *Cancer Res* 2001;61:7122–9.
- Jiang M, Shao ZM, Wu J, et al. p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. *Int J Cancer* 1997;74:529–34.
- Pruneri G, Pignataro L, Carboni N, et al. Clinical relevance of expression of the CIP/KIP cell-cycle inhibitors p21 and p27 in laryngeal cancer. *J Clin Oncol* 1999;17:3150–9.
- Xi YG, Ding KY, Su XL, et al. p53 polymorphism and p21WAF1/CIP1 haplotype in the intestinal gastric cancer and the precancerous lesions. *Carcinogenesis* 2004;25:2201–6.
- Li G, Liu Z, Sturgis EM, et al. Genetic polymorphisms of p21 are associated with risk of squamous cell carcinoma of the head and neck. *Carcinogenesis* 2005;26:1596–602.
- Boley SE, Wong VA, French JE, Recio L. p53 heterozygosity alters the mRNA expression of p53 target genes in the bone marrow in response to inhaled benzene. *Toxicol Sci* 2002;66:209–15.
- 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.
- Wu F, Zhang Z, Wan J, et al. Genetic polymorphisms in hMTH1, hOGG1 and hMYH and risk of chronic benzene poisoning in a Chinese occupational population. *Toxicol Appl Pharmacol* 2008;233:447–53.
- Sun P, Qian J, Zhang ZB, 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.
- Dosemeci M, Yin SN, Linet M, et al. Indirect validation of benzene exposure assessment by association with benzene poisoning. *Environ Health Perspect* 1996;104 Suppl 6:1343–7.
- Wu X, Zhao H, Amos CI, et al. p53 genotypes and haplotypes associated with lung cancer susceptibility and ethnicity. *J Natl Cancer Inst* 2002;94:681–90.
- Facher EA, Becich MJ, Deka A, Law JC. Association between human cancer and two polymorphisms occurring together in the p21Waf1/Cip1 cyclin-dependent kinase inhibitor gene. *Cancer* 1997;79:2424–9.
- Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005;15:97–8.
- Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 2001;68:978–89.
- Stephens M, Scheet P. Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. *Am J Hum Genet* 2005;76:449–62.
- Boley SE, Wong VA, French JE, Recio L. p53 heterozygosity alters the mRNA expression of p53 target genes in the bone marrow in response to inhaled benzene. *Toxicol Sci* 2002;66:209–15.
- Yoon BI, Li GX, Kitada K, et al. Mechanisms of benzene-induced hematotoxicity and leukemogenicity: cDNA microarray analyses using mouse bone marrow tissue. *Environ Health Perspect* 2003;111:1411–20.
- Ohayon T, Gershoni-Baruch R, Papa MZ, Distelman Menachem T, Eisenberg Barzilai S, Friedman E. The R72P P53 mutation is associated with familial breast cancer in Jewish women. *Br J Cancer* 2005;92:1144–8.
- Cox DG, Deer D, Guo Q, et al. The p53 Arg72Pro and MDM2 -309 polymorphisms and risk of breast cancer in the nurses' health studies. *Cancer Causes Control* 2007;18:621–5.
- Szymanowska A, Jassem E, Dziadziuszko R, et al. Increased risk of non-small cell lung cancer and frequency of somatic TP53 gene mutations in Pro72 carriers of TP53 Arg72Pro polymorphism. *Lung Cancer* 2006;52:9–14.
- Shen M, Lan Q, Zhang L, et al. Polymorphisms in genes involved in DNA double-strand break repair pathway and susceptibility to benzene-induced hematotoxicity. *Carcinogenesis* 2006;27:2083–9.
- Lehman TA, Haffty BG, Carbone CJ, et al. Elevated frequency and functional activity of a specific germ-line p53 intron mutation in familial breast cancer. *Cancer Res* 2000;60:1062–9.
- Gemignani F, Moreno V, Landi S, et al. A TP53 polymorphism is associated with increased risk of colorectal cancer and with reduced levels of TP53 mRNA. *Oncogene* 2004;23:1954–6.
- Fischer-Maliszewska L, Kazanowska B, Kusnierczyk P, et al. Is p53 intronic variant G13964C associated with predisposition to cancer? *J Appl Genet* 2003;44:547–52.
- Buyru N, Tezol A, Dalay N. p53 intronic G13964C variant in colon cancer and its association with HPV. *Anticancer Res* 2005;25:2767–9.
- Fan R, Wu MT, Miller D, et al. The p53 codon 72 polymorphism and lung cancer risk. *Cancer Epidemiol Biomarkers Prev* 2000;9:1037–42.
- Yu MW, Yang SY, Chiu YH, Chiang YC, Liaw YF, Chen CJ. A p53 genetic polymorphism as a modulator of hepatocellular carcinoma risk in relation to chronic liver disease, familial tendency, and cigarette smoking in hepatitis B carriers. *Hepatology* 1999;29:697–702.
- Wang YC, Lee HS, Chen SK, Chang YY, Chen CY. Prognostic significance of p53 codon 72 polymorphism in lung carcinomas. *Eur J Cancer* 1999;35:226–30.
- Shen H, Zheng Y, Sturgis EM, Spitz MR, Wei Q. P53 codon 72 polymorphism and risk of squamous cell carcinoma of the head and neck: a case-control study. *Cancer Lett* 2002;183:123–30.

44. Su L, Sai Y, Fan R, et al. P53 (codon 72) and P21 (codon 31) polymorphisms alter *in vivo* mRNA expression of p21. *Lung Cancer* 2003;40:259–66.
45. Kibel AS, Suarez BK, Belani J, et al. CDKN1A and CDKN1B polymorphisms and risk of advanced prostate carcinoma. *Cancer Res* 2003;63:2033–6.
46. Chen WC, Wu HC, Hsu CD, Chen HY, Tsai FJ. p21 gene codon 31 polymorphism is associated with bladder cancer. *Urol Oncol* 2002;7:63–6.
47. Keshava C, Frye BL, Wolff MS, McCanlies EC, Weston A. Waf-1 (p21) and p53 polymorphisms in breast cancer. *Cancer Epidemiol Biomarkers Prev* 2002;11:127–30.
48. Huang SP, Wu WJ, Chang WS, et al. p53 Codon 72 and p21 codon 31 polymorphisms in prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2004;13:2217–24.
49. Mucci LA, Wedren S, Tamimi RM, Trichopoulos D, Adami HO. The role of gene-environment interaction in the aetiology of human cancer: examples from cancers of the large bowel, lung and breast. *J Intern Med* 2001;249:477–93.
50. Seiji K, Jin C, Watanabe T, Nakatsuka H, Ikeda M. Sister chromatid exchanges in peripheral lymphocytes of workers exposed to benzene, trichloroethylene, or tetrachloroethylene, with reference to smoking habits. *Int Arch Occup Environ Health* 1990;62:171–6.

Association of Genetic Polymorphisms, mRNA Expression of *p53* and *p21* with Chronic Benzene Poisoning in a Chinese Occupational Population

Pin Sun, Yulan Qiu, Zhongbin Zhang, et al.

Cancer Epidemiol Biomarkers Prev 2009;18:1821-1828.

Updated version Access the most recent version of this article at:
<http://cebp.aacrjournals.org/content/18/6/1821>

Cited articles This article cites 49 articles, 11 of which you can access for free at:
<http://cebp.aacrjournals.org/content/18/6/1821.full#ref-list-1>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cebp.aacrjournals.org/content/18/6/1821>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.