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Large-scale evaluation of candidate genes identifies associations between DNA repair and genomic maintenance and development of benzene hematotoxicity

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Benzene is an established human hematotoxicant and leukemogen but its mechanism of action is unclear. To investigate the role of single-nucleotide polymorphisms (SNPs) on benzene-induced hematotoxicity, we analyzed 1395 SNPs in 411 genes using an Illumina GoldenGate assay in 250 benzene-exposed workers and 140 unexposed controls. Highly significant findings clustered in five genes (*BLM*, *TP53*, *RAD51*, *WDR79* and *WRN*) that play a critical role in DNA repair and genomic maintenance, and these regions were then further investigated with tagSNPs. One or more SNPs in each gene were associated with highly significant 10–20% reductions (*P* values ranged from 0.0011 to 0.0002) in the white blood cell (WBC) count among benzene-exposed workers but not controls, with evidence for gene–environment interactions for SNPs in *BLM*, *WRN* and *RAD51*. Further, among workers exposed to benzene, the genotype-associated risk of having a WBC count <4000 cells/μl increased when using individuals with progressively higher WBC counts as the comparison group, with some odds ratios >8-fold. *In vitro* functional studies revealed that deletion of *SGS1* in yeast, equivalent to lacking BLM and WRN function in humans, caused reduced cellular growth in the presence of the toxic benzene metabolite hydroquinone, and knockdown of *WRN* using specific short hairpin RNA increased susceptibility of human TK6 cells to hydroquinone toxicity. Our findings suggest that SNPs involved in DNA repair and genomic maintenance, with particular clustering in the homologous DNA recombination pathway, play an important role in benzene-induced hematotoxicity.

Introduction

There is substantial interindividual variation in sensitivity to the toxic effects of chemicals, including drugs and industrial compounds. For example, there is evidence of striking variation in benzene toxicity among workers with comparable occupational exposure (1). The reasons underlying this variation are unknown, but studies to date have identified a small number of single-nucleotide polymorphisms (SNPs) in candidate genes that appear to confer susceptibility to benzene hematotoxicity (1–5).

Benzene is a ubiquitous environmental pollutant and its environmental regulation is of great economic importance because it is a component of automobile exhaust, gasoline and cigarette smoke. Chronic exposure to benzene induces chromosome damage, bone marrow depression and a reduction in the number of circulating peripheral blood cells (1,6–10). Occupational exposure to benzene has been associated with increased risks of aplastic anemia, myelodysplastic syndrome, leukemia and lymphoma (11). Quantitative understanding of human susceptibility to this toxic pollutant and the role of genetic variation needs to be elucidated.

We have previously demonstrated that benzene exposure produced significant declines in white blood cell (WBC) counts at lower levels than previously shown and that this association was modified by SNPs in several candidate genes (1–5). In the present study, we used the Illumina® GoldenGate assay to genotype a set of common SNPs to broadly evaluate additional sources of potential genetic contributors to benzene hematotoxicity. Candidate SNPs in genes that play an important role in a wide range of pathways important in carcinogenesis were drawn from the SNP500Cancer database (http://snp500cancer.nci.nih.gov). A total of 1395 SNPs in 411 genes were successfully genotyped. Highly significant findings clustered in genes (*BLM*, *TP53*, *RAD51*, *WDR79* and *WRN*) that play a critical role in DNA repair and genomic maintenance. These regions were further investigated with tagSNPs, and *in vitro* functional studies in yeast and human TK6 cells were pursued to provide corroborative evidence to support notable associations.

Abbreviations: FDR, false discovery rate; HR, homologous recombination; ln, natural log; shRNA, short hairpin RNA; SNP, single-nucleotide polymorphism; WBC, white blood cell.

Materials and methods

Study population, exposure assessment and biological sample collection

The study population and methods have previously been described (1,12). In brief, we enrolled 250 workers from shoe factories that use benzene-containing compounds in the manufacturing process and 140 unexposed controls from Tianjin, China. Controls were frequency matched to exposed workers by sex and age. Extensive air monitoring using 3M™ organic vapor monitors, measurement of urinary benzene and assessment of dermal exposure to benzene were used in the study's exposure assessment component (1,12). The study was approved by the Institutional Review Boards of the U.S. National Cancer Institute and the China Center for Disease Control. Participation was voluntary, and written informed consent was provided by all study participants. The participation rate was ∼95%.

Subjects were administered a detailed questionnaire requesting information on lifetime occupational and environmental history and genotoxic exposures, recent infections medical history, tobacco smoking and alcohol intake. Interviews, physical exams and biological sample collection took place in June 2000 (88 workers) and in May and June 2001 (remaining workers). Blood samples were collected from study subjects and delivered to the lab within 6 h. The complete blood count and differential were analyzed by a Beckman-Coulter T540® blood counter, and the major lymphocyte subsets were analyzed by a Becton Dickinson FACSCalibur™ flow cytometer (Software: SimuISET v3.1) (1). Twenty-eight subjects were enrolled in both years (supplementary Table 1 is available at *Carcinogenesis* Online).

Genotyping

Genotyping was performed on a GoldenGate assay (Illumina, http://www.illumina. com) using SNPs in the SNP500Cancer project (http://snp500cancer.nci.nih.gov that were validated in individuals from four ethnic groups, one of which was comprised of individuals from the Pacific Rim region, 74% of whom were from eastern Asian countries. These genes were selected based on previous resequence analyses and evidence relating to carcinogenic processes (13). Based on an initial screen, 1536 SNPs were selected from 3072 candidate SNPs using the GoldenGate assay from the SNP500Cancer database and were subsequently analyzed in the HapMap Centre d'Etude du Polymorphisme Humain Utah samples. These SNPs are functional or probably functional, have demonstrated a previous association with cancer or other diseases or were used to extend genomic coverage. Of the 1536 SNPs, 141 SNPs were subsequently excluded due to a low minor allele frequency (<1%) or unstable assays, so 1395 SNPs in or near 411 genes were included in this report. Sixty-eight blinded duplicate samples were randomly interspersed throughout the study sample plates and showed intrasubject concordance rates >99% for all

assays. Completion rates were ≥99% for 98.5% of the assays. Of these 1395 SNPs, 193 were in 51 genes in DNA repair and genome maintenance, 191 were in 50 genes in cell cycle pathways, 216 were in 69 genes in cytokine-related pathways, 28 were in 10 genes in one-carbon metabolism, 138 were in 43 genes in signal transduction, 30 were in 5 genes in telomere maintenance, 107 were in 32 genes in transport activity, 111 were in 31 genes in xenobiotic metabolism, 210 SNPs were in 60 genes in other metabolic pathways and 171 were in 60 genes in miscellaneous pathways. Of the 1395 SNPs successfully analyzed on the Oligo Pool (supplementary Table 2 is available at *Carcinogenesis* Online), 34 were published previously as TaqMan assays (22 SNPs in ref. 2; 12 SNPs in ref. 4).

Additional genotyping was performed to investigate common SNPs in the five genes (*WRN*, *BLM*, *RAD51*, *TP53* and *WDR79*) that clustered in DNA repair and genomic maintenance using SNPlex (ABI, Foster City, CA) and TaqMan (ABI). The International HapMap using Tagzilla samples was used to select tagging SNPs for an Eastern Asian population based on the following criteria: (i) minor allele frequency >5%; (ii) r^2 >0.8 was used as the cutoff point and (iii) SNPs with a design score of 1.1 were weighted higher and SNPs with a design score <0.6 were excluded. In addition, data previously genotyped by TaqMan in these genes (4) were included in the analysis. A complete list of the SNPs for each of the five genes is presented in supplementary Table 3 (available at *Carcinogenesis* Online). In total, 38 SNPs in *BLM*, 38 SNPs in *WRN*, 9 SNPs in *TP53*, 3 SNPs in *WDR79* and 14 SNPs in *RAD51* were available for analysis. Of these, one SNP in *TP53* and six SNPs in *WRN* were previously reported (4) and are identified as such in Figure 1, Figure 2 and/or supplementary Tables 3–5 (available at *Carcinogenesis* Online). Data were available for a variable number of subjects because of inadequate amounts of DNA available on one or more platforms or completion rates under 100% for particular assays.

Statistical analysis

Tests for fitness for Hardy–Weinberg equilibrium were analyzed for all subjects using a Pearson χ^2 test, with one degree of freedom. The genotype frequencies for 5% of the SNPs were not in Hardy–Weinberg equilibrium ($P < 0.05$), consistent with chance. Quality control data were rechecked for all assays not in Hardy–Weinberg equilibrium and genotype data were confirmed.

Previous studies have linked repeated WBC counts under 4000 cells/μl to risk of developing hematologic malignancies and related disorders among workers exposed to benzene (3,14,15), providing support for the relevance of the WBC count as an important intermediate endpoint in molecular epidemiology studies of benzene. We therefore first examined the effect of each SNP on total WBC count among workers exposed to benzene to screen for highly significant associations. We then determined if these associations were limited to workers exposed to benzene, or if they were also present among unexposed controls, and tested for interaction. For each analysis, the most prevalent homozygous genotype was used as the reference. If minor allele homozygotes or heterozygotes contained fewer than five subjects, then the two groups were combined in the analysis.

Because the WBC count is a continuous variable, the relationship between each genotype and natural log (ln) of the WBC count was evaluated using linear regression adjusting for age (continuous), sex, current cigarette smoking status (yes/no), current alcohol consumption (yes/no), recent infections (yes/no) and body mass index. For analyses restricted to benzene-exposed workers, the model was also adjusted for the ln mean air benzene and ln mean air toluene exposure in the month prior to phlebotomy (1). Tests for trends were conducted assuming a dose-response relationship with increasing number of variant alleles (i.e. 0, 1 and 2 according to the number of variant alleles). Gene–benzene interactions were tested by introducing an interaction term between the genotype (variant homozygous and heterozygous carriers combined versus most prevalent homozygous carriers) and benzene exposure (yes/no) into each model.

There are up to 418 observations on 390 unique subjects (140 controls; 250 benzene-exposed workers, of whom 28 were studied in both 2000 and 2001). Data from the 28 benzene-exposed workers studied in both enrollment years were treated as independent observations by using Generalized Estimating Equations to adjust for a potential correlation between the repeated measurements (16). Results were very similar when data from only the first or second year of the study were used for these 28 subjects.

The false discovery rate (FDR) using the Benjamini–Hochberg (17) method was used to take into account testing of multiple hypotheses, with a value of 0.05 used to identify the most noteworthy associations. The change in WBC counts of homozygous carriers of the rare allele versus the common allele was used to calculate the FDR values. This comparison provides the maximum contrast for effects across genotypes. The effects of SNPs with FDR values <0.05 were further tested on specific WBC subtypes among benzene-exposed workers. All *P* values that are presented are two sided, and all analyses were carried out using SAS version 8.02 software (SAS Institute, Cary, NC).

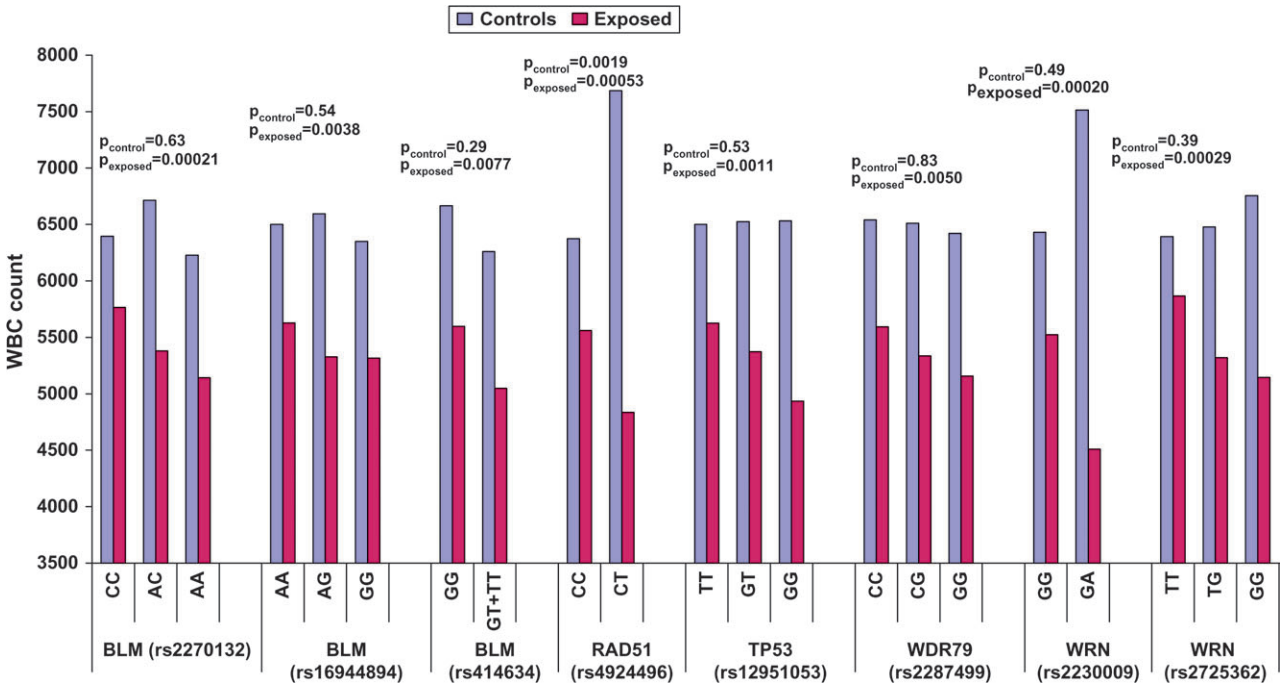

Fig. 1. Effect of SNPs in *BLM* (rs401549), *RAD51* (rs11852786), *TP53* (rs1042522), *WDR79* (rs2287499) and *WRN* (rs2230009 and rs2725362) and peripheral WBC counts in workers exposed to benzene and controls. Tests for trends were conducted assuming a dose-response relationship with increasing number of variant alleles (i.e. 0, 1 and 2 according to the number of variant alleles). The *WRN* SNPs rs2230009 and rs2725362 were published previously (4). Models were adjusted for age, sex, current smoking, current alcohol drinking, body mass index, recent infections (flu or respiratory infections, in the previous month) and among exposed workers ln air benzene exposure and ln air toluene exposure in the month before phlebotomy. There are two controls without body mass index data and they are excluded from the statistical analysis.

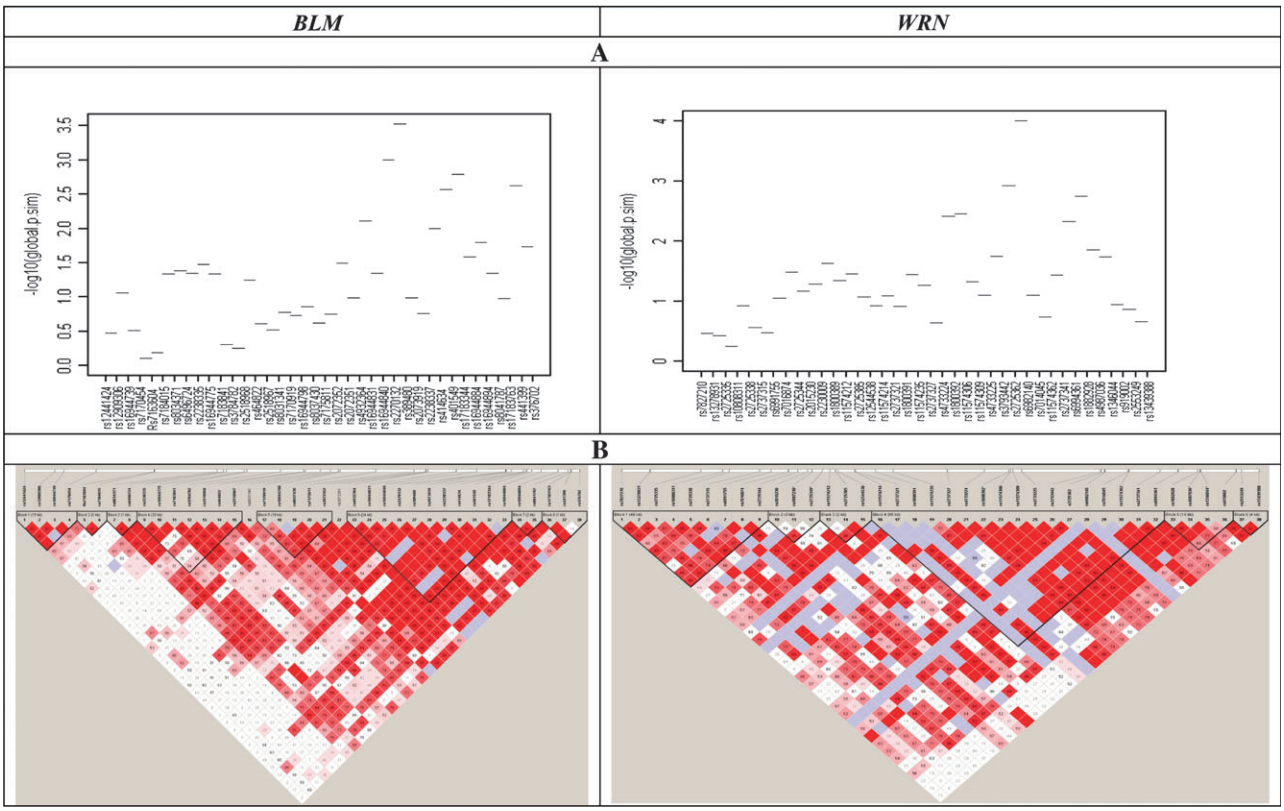

Fig. 2. (**A**) Two-SNP sliding window haplotype analysis of WBC count/μl blood using UNPHASED (−log *P*-values), for *BLM* and *WRN* with nominal haplotype *P*-values <0.05. Sliding windows run 5′ to 3′ in SNP order. The window numbers are the same as the number of the first SNP within each two-SNP set. SNP numbers for *BLM* and *WRN* genes can be found in supplementary Table 3 (available at *Carcinogenesis* Online). The following *WRN* SNPs were published previously (4): rs2230009, rs2725362, rs1346044, rs1800389, rs1800392 and rs4987036. (**B**) Color scheme is based on D' and logarithm of the odds of linkage (LOD) score values: white $D' < 1$ and LOD < 2, gray-blue $D' = 1$ and LOD < 2, shades of pink/red: $D' < 1$ and LOD ≥ 2 and bright red $D' = 1$ and LOD ≥ 2. Numbers in squares are D' values (values of 1.0 are not shown). Block definition is based on solid spine of linkage disequilibrium method with a minimum frequency of 0.05 for the fourth gamete (50).

Risk (odds ratios with 95% confidence intervals) of having a WBC count <4000/μl compared with subjects with WBC counts ≥4000, ≥5000, ≥6000 and ≥7000/μl blood for benzene-exposed subjects in the variant heterozygous and homozygous genotyping categories versus common homozygous genotyping category was analyzed by unconditional logistic regression, with adjustment for potential confounders described previously. Trend analysis was carried assuming a dose-response relationship with increasing number of variant alleles (i.e. 0, 1 and 2 according to the number of variant alleles). Haplotype block structure was examined with HaploView (http://www.broad.mit.edu/personal/jcbarret/haploview/) using the solid spine linkage disequilibrium method with a minimum frequency of 0.05 for the fourth gamete. Haplotype frequencies for genes showing blocks of linkage disequilibrium were estimated using the expectation–maximization algorithm (18). The association between estimated haplotypes and blood cell counts was assessed using the HaploStats program in R (Version 2.0.1) (19), adjusting for potential confounders.

We also used a sliding window two-SNP haplotype approach to comprehensively evaluate potentially important loci in small genetic regions that may have been overlooked with the single-locus analysis (20). Haplotypes were estimated using an expectation–maximization algorithm (18) and a global score test (21) was used to determine the significance of the association for each window using the software program, HaploStats.

Functional studies in yeast

Two genes with the strongest associations with WBC count, *BLM* and *WRN*, are DNA helicases and play a critical role in DNA repair. To evaluate the impact of loss of DNA helicase activity on cell growth in the presence of a key benzene toxic metabolite, hydroquinone, we evaluated growth curves of wild-type yeast strain and *sgs1*Δ, which does not have a functional DNA helicase system and is a model system used to explore the basis of Bloom and Werner syndromes (22). Exponentially growing cultures of yeast wild-type and *sgs1*Δ in rich media were diluted to an optical density at 595 nm of 0.0165 and inoculated in a 48-well microplate with increasing concentrations of hydroquinone. Plates were incubated in a Tecan Genios spectrophotometer set to 30°C and intermittent shaking, with optical density at 595 nm measurements taken at 15 min intervals for 24 h. For each strain, the raw optical density at 595 nm data were averaged for all replicate wells, background corrected and plotted as a function of time. The area under the curve was calculated for each of the hydroquinone treatments as a measure of cell growth and normalized as a percentage of the control for comparison.

Cell viability assay using TK6 cell lines with a stably suppressed WRN protein level

WRN-specific short hairpin RNA (shRNA) (shWRN) was designed using Invitrogen's BLOCK-iT RNAi designer and human TK6 cells were transduced with either shWRN or universal non-specific shRNA using a lentiviral vector. After antibiotic selection, single clones with suppressed WRN expression were identified and expanded. The sequences of shWRN and universal non-specific shRNA used in the assays are available upon request. TK6 cells with shWRN or universal non-specific shRNA control were seeded in a concentration of 100 000 cells/ml and maintained in RPMI medium containing 10% fetal bovine serum and antibiotic solution (100 μg/ml penicillin and 100 IU/ml streptomycin) at 37°C in a 5% CO_2 incubator for 24 h. After this period, the cells were treated to 5, 10 and 20 μm hydroquinone for 24 h. Total number of cells was counted using a hemocytometer with the trypan blue exclusion assay in unexposed cultures and in those treated with hydroquinone, and the cell viability was calculated and normalized as a percentage of the control for comparison.

Results

Characteristics of the study subjects

Study subjects were young adults (mean ± SD: 30 ± 8 years) in both the exposed and control subjects; 66% of the exposed subjects and

63% of the controls were females. Current smoking status, recent infection history, current alcohol use and body mass index measurements were similar between the exposed and control subjects. Personal benzene air measurements collected during the month before phlebotomy (two measurements per subject on average; mean ± SD: 5.4 ± 12.1 p.p.m.), which were the primary approach to exposure assessment in this study, were highly correlated ($r = 0.88$, $P < 0.0001$) with urine benzene levels (mean ± SD: 158 ± 536 μg/l). Compared with controls, the total WBC count, major WBC subtypes and the platelet count were significantly decreased among benzene-exposed workers (supplementary Table 1 is available at *Carcinogenesis* Online).

Initial single-SNP analyses

The initial screening of 1395 SNPs in 411 genes (supplementary Table 2 is available at *Carcinogenesis* Online) identified one or more SNPs in 15 genes that were strongly associated with altered WBC counts among workers exposed to benzene, after accounting for multiple comparisons (FDR values <0.05) (Table I). Six of the 15 genes had been previously identified in candidate gene studies genotyped by TaqMan, including myeloperoxidase which can activate benzene metabolites to toxic intermediates, and several cytokine genes involved in the immune response. The most significantly associated genes were apolipoprotein B and insulin-like growth factor 2 receptor. However, it was particularly noteworthy that of the 15 genes identified, five (33%) (*BLM*, *TP53*, *RAD51*, *WDR79 and WRN*) clustered in DNA repair and genomic maintenance. In contrast, only 12% of the 411 genes analyzed in the Oligo Pool are part of this pathway ($P = 0.01$ for difference). This suggested that the DNA repair and genomic maintenance pathway plays an important role in benzene-induced hematotoxicity. We therefore focused our attention on these genes and carried out additional genotyping to more comprehensively assess genetic variation in these loci.

Expanded coverage of SNPs in DNA repair and genomic maintenance genes

In order to increase coverage within each of the five gene regions for this Eastern Asian population, additional SNPs were analyzed as tagSNPs (total 102 SNPs: 38 for *BLM*, 38 for *WRN*, 14 for *RAD51*, 9 for *TP53* and 3 for *WDR79*). Twelve SNPs in *BLM*, nine SNPs in *WRN*, two SNPs in *RAD51* and six SNPs in *TP53–WDR79* were significantly associated with a decreased WBC count among exposed workers (supplementary Table 3 is available at *Carcinogenesis* Online). Among the benzene-exposed workers, WBC counts decreased ~10% for the minor allele of most SNPs, with changes approaching a 20% decline for some SNPs. In contrast, the vast majority of the most noteworthy SNPs among benzene-exposed workers had a negligible impact on WBC counts among controls (supplementary Table 3 is available at *Carcinogenesis* Online).

To assess linkage disequilibrium between notable SNPs in a gene, we evaluated both D' and r^2 to narrow the number of SNPs not strongly correlated before employing an analysis plan of multivariable linear regression analysis to identify SNPs with an independent influence on total WBC count. We identified three SNPs in *BLM* (rs2270132, rs414634 and rs16944894; r^2 values were <0.2 for each pairwise comparison), two SNPs in *WRN* (rs2230009 and rs2725362; $r^2 = 0.03$), one SNP in *RAD51* (rs4924496), one SNP in *TP53* (rs12951053) and one in *WDR79* (rs2287499) that were most strongly and independently associated with WBC count (Figure 1) among all SNPs genotyped by each platform in these loci. Although we expanded genomic coverage from ~25% in our previous report with six *WRN* SNPs (4) to 97% in the current report with the addition of 32 SNPs, the two most informative SNPs in *WRN* were those that we previously published, noted above (4). In contrast, when we expanded genomic coverage from 12% in the *TP53* gene region (4) to 77% with the addition of 12 SNPs in *TP53* and the adjacent *WDR79*, which is in the same linkage block, we identified a new SNP in *TP53*, noted above, which was more statistically significantly associated with a decline in WBC counts and subsets than the previously reported finding in this region (4) (supplementary Tables 3–5 are available at *Carcinogenesis* Online). Among benzene-exposed workers, there were highly statistically significant associations with a decline in WBC count. In contrast, among the controls, the WBC counts varied little by genotype for most SNPs, and some SNPs were associated with an increased WBC count (Figure 1). Tests for interaction were significant for SNPs in *WRN* (rs2725362, $P = 0.021$) and *RAD51* (rs4924496, $P < 0.0001$) and of borderline significance for a SNP in *BLM* (rs2270132, $P = 0.068$), suggesting gene–environment interactions of these SNPs with benzene exposure. We further explored the effects of these genotypes among workers exposed to <1 p.p.m. benzene, the current USA 8 h permissible exposure level in the workplace, finding that WBC counts were significantly decreased for individuals carrying the variant alleles of both *WRN* SNPs (rs2230009, $P = 0.00020$; rs2725362, $P = 0.00029$). This suggests a critical role for the helicase WRN in conferring susceptibility to benzene-induced hematotoxicity at even low levels of exposure.

We conducted further analyses to evaluate the influence of these SNPs on specific WBC subpopulations in benzene-exposed workers (Table II, supplementary Tables 4 and 5 are available at *Carcinogenesis* Online). The effect of SNPs in *BLM* (rs2270132 and rs16944894) displayed the broadest effect on WBC subtypes, with significant decreases for granulocytes, total lymphocyte count, $CD4^+$-T cells, $CD8^+$-T cells, B cells and monocytes. In contrast, the effect of *BLM* (rs414634) was limited to granulocytes. SNPs in *RAD51* (rs4924496) and *WRN* (rs2725362) were significantly associated with decreased granulocytes, total lymphocytes, $CD4^+$-T cells and $CD8^+$-T cells. The *TP53* SNP (rs1042522) was associated with decreased granulocytes, $CD4^+$-T cells and B cells, and the *WDR79* SNP (rs17885803) was associated with a decline in granulocytes and $CD4^+$-T cells.

We have previously shown that having a total WBC count <4000/μl measured repeatedly over several months, a compensable condition in China called benzene poisoning, is associated with increased risk of subsequently developing a hematological malignancy or related disorder (3,15). As such, we were interested in exploring, among workers exposed to benzene, genotype risks associated with a WBC count <4000/μl in comparison with referent subjects with a WBC count ≥4000/μl, as well as subjects with a progressively higher WBC count phenotype. Odds ratios became progressively larger when using individuals with more extreme, higher WBC counts as the

Table I. Raw *P* values and FDR values for the most highly statistically significant SNP associations with peripheral WBC count among benzene-exposed workers in Tianjin, China[a]

SNP name	rs number	Base pair position	Raw *P* values	FDR values
APOB	rs3791981	IVS18+336T>C	0.0000088	0.0098
IGF2R	rs1570070	Ex9+5A>G	0.000015	0.0098
IL1A	rs17561	Ex5+21G>T	0.000063	0.020
GSK3B	rs1719888	IVS10+3386G>A	0.000076	0.020
WRN[b]	rs2230009	Ex4−16G>A	0.00020	0.020
TP53	rs12951053	IVS7+92T>G	0.00040	0.026
GPX3	rs8177426	IVS1−1961A>G	0.00040	0.026
RXRA	rs1805352	IVS2−46C>A	0.00048	0.027
BLM	rs2270132	IVS19−499A>C	0.00050	0.027
CSF3	rs3917979	IVS9−145A>G	0.00050	0.027
RAD51	rs4924496	IVS3+1932T>C	0.00053	0.027
EFNB3	rs3744262	Ex5−929G>A	0.00069	0.034
IL10[b]	rs1800871	−853C>T	0.00090	0.041
MPO	rs2071409	IVS11−6A>C	0.00092	0.041
WDR79	rs17885803	IVS1−60C>T	0.0011	0.047

[a]*P*-value from test of change in WBC count in homozygous carriers of the rare allele versus the common allele.
[b]These SNPs were genotyped in this study as part of an earlier candidate genotyping project and reported previously (2,4).

Table II. Effect on WBC subtypes of SNPs in four genomic maintenance and DNA repair genes among benzene-exposed subjects

Base pair position		Granulocytes			Lymphocytes		CD4$^+$ T cells		CD8$^+$ T cells		B cells		NK cells		Monocytes	
		n	Mean ± SD[a]	*P*[b]	Mean ± SD[a]	*P*[b]	Mean ± SD[a]	*P*[b]	Mean ± SD[a]	*P*[b]	Mean ± SD[a]	*P*[b]	Mean ± SD[a]	*P*[b]	Mean ± SD[a]	*P*[b]
BLM	CC	102	3500 ± 1077		2019 ± 559		670 ± 170		575 ± 215		184 ± 95		545 ± 286		232 ± 101	
rs2270132	AC	124	3260 ± 969	0.08	1865 ± 491	**0.02**	590 ± 188	**0.00025**	532 ± 207	0.06	158 ± 81	**0.0098**	531 ± 270	0.66	204 ± 84	**0.02**
IVS19−499C>A	AA	44	3059 ± 1056	**0.0012**	1920 ± 499	0.06	587 ± 166	**0.0036**	537 ± 229	0.08	184 ± 93	0.73	568 ± 297	0.73	202 ± 95	**0.05**
	AA+AC	168	3207 ± 993	**0.01**	1880 ± 492	**0.01**	590 ± 182	**0.000043**	533 ± 212	**0.03**	165 ± 84	**0.03**	541 ± 277	0.62	204 ± 87	**0.0086**
Trend				**0.0013**		**0.02**		**0.00027**		**0.04**		0.24		0.66		**0.02**
BLM	GG	209	3411 ± 1060		1967 ± 533		631 ± 189		550 ± 214		178 ± 92		558 ± 285		219 ± 98	
rs414634	GT	59	3000 ± 884	**0.02**	1837 ± 457	0.26	592 ± 160	0.24	562 ± 219	0.61	159 ± 77	0.51	477 ± 250	0.32	202 ± 73	0.44
IVS21+1617A>C	TT	2														
	TT+GT	61	3011 ± 872	**0.02**	1836 ± 468	0.20	593 ± 161	0.23	561 ± 219	0.62	159 ± 76	0.49	480 ± 254	0.28	200 ± 73	0.32
Trend				**0.02**		0.18		0.25		0.65		0.47		0.25		0.22
BLM	AA	153	3404 ± 1057		1995 ± 546		654 ± 179		579 ± 210		182 ± 91		538 ± 287		228 ± 96	
rs16944894	AG	92	3270 ± 1037	0.10	1863 ± 461	**0.0079**	580 ± 185	**0.00052**	524 ± 225	**0.0025**	157 ± 81	**0.0063**	550 ± 266	0.92	195 ± 79	**0.0013**
4793 bp 3′ of ST	GG	19	3126 ± 880	**0.04**	1968 ± 529	0.34	590 ± 129	0.07	504 ± 199	0.07	210 ± 89	0.26	603 ± 291	0.84	221 ± 108	0.99
	GG+AG	111	3245 ± 1009	0.05	1881 ± 473	**0.0075**	582 ± 176	**0.00031**	521 ± 220	**0.00090**	166 ± 85	**0.03**	559 ± 270	0.98	199 ± 85	**0.0046**
Trend				**0.03**		**0.02**		**0.00082**		**0.0016**		0.31		0.94		0.07
RAD51	CC	234	3385 ± 1061		1958 ± 519		634 ± 177		558 ± 222		174 ± 87		549 ± 276		218 ± 93	
rs4924496	CT	36	2881 ± 695	**0.0018**	1767 ± 516	**0.04**	526 ± 188	**0.0019**	490 ± 131	0.07	159 ± 103	0.11	500 ± 302	0.20	189 ± 89	**0.05**
IVS3+1932T>C																
TP53[c]	TT	149	3451 ± 1140		1956 ± 547		632 ± 185		551 ± 220		181 ± 95		538 ± 266		218 ± 91	
rs12951053	GT	92	3202 ± 903	0.12	1952 ± 492	0.99	623 ± 179	0.82	567 ± 208	0.34	168 ± 81	0.22	556 ± 281	0.96	217 ± 97	0.72
IVS7+92T>G	GG	29	3000 ± 703	**0.0040**	1748 ± 451	**0.01**	548 ± 160	**0.0085**	483 ± 191	0.10	142 ± 76	**0.0069**	519 ± 345	0.29	186 ± 92	0.08
	GG+GT	121	3154 ± 861	**0.02**	1903 ± 489	0.37	605 ± 177	0.27	547 ± 207	0.88	162 ± 80	**0.04**	547 ± 297	0.59	210 ± 96	0.33
Trend				**0.0046**		0.08		**0.05**		0.44		**0.0083**		0.37		0.14
WDR79[c]	CC	148	3426 ± 1111		1951 ± 544		630 ± 183		553 ± 222		178 ± 97		540 ± 273		216 ± 92	
rs2287499	CG	103	3198 ± 970	**0.045**	1925 ± 497	0.68	624 ± 185	0.44	556 ± 211	0.67	166 ± 80	0.14	525 ± 266	0.59	212 ± 92	0.79
Ex1−230C>G	GG	19	3121 ± 568	**0.030**	1821 ± 485	0.08	523 ± 123	**0.0040**	483 ± 151	0.12	157 ± 65	0.15	648 ± 382	0.74	216 ± 112	0.56
	GG+CG	122	3186 ± 917	**0.020**	1909 ± 494	0.38	608 ± 180	0.15	545 ± 204	0.96	165 ± 77	0.09	544 ± 289	0.72	212 ± 95	0.67
Trend				**0.010**		0.17		**0.029**		0.51		0.07		0.92		0.58

Note. NK, natural killer.

[a]Unadjusted cell counts/μl blood as mean ± SD. Complete blood cell counts and differentials were analyzed with a Beckman-Coulter® T540 blood counter. Lymphocyte subsets were measured with a Becton Dickinson FACSCalibur™ flow cytometer (Software: SimulSET v3.1).

[b]Linear regression was used to test for differences between cell counts in each specified genotype group versus subjects homozygous for the common allele. Models were adjusted for age, sex, current smoking, current alcohol drinking, body mass index, recent infections, ln air benzene exposure and ln air toluene exposure in the month before phlebotomy. *P* values <0.05 are bolded.

[c]$D' = 0.81$ $r^2 = 0.33$ between rs12951053 and rs2287499.

comparison group (e.g. for *BLM* rs2270132$_{AA + AC}$, odds ratios and 95% confidence intervals were 3.2 (1.2–8.5), 5.0 (1.7–14), 7.5 (2.2–25) and 8.6 (1.7–42) for risk of having a WBC count <4000 versus ≥4000, ≥5000, ≥6000 and ≥7000 cells/μl, respectively (Table III).

Sliding window haplotype analyses

Haplotype analysis of *RAD51* and the *TP53/WDR79* gene region showed that there was no evidence of an effect beyond the single SNP shown in Figure 1 and Table II. Initial analysis suggested that two SNPs in *WRN* and three SNPs in *BLM* were independently associated with WBC count. To prioritize regions of interest of each of the two genes and to identify other regions that might have a stronger association than the effect for individual SNPs, we used a sliding window method to construct successive and adjacent haplotypes across *BLM* and *WRN* in windows of two adjacent SNPs (Figure 2). Haplotype analyses were carried out for 38 SNPs in both *BLM* and *WRN*. We found that one region for *BLM* and two regions for *WRN* yielded strong evidence for association with WBC counts. The peak region for *BLM* included all three significant SNPs (rs2270132, rs414634 and rs16944894) shown in supplementary Table 3 (available at *Carcinogenesis* Online) (Figure 2). Detailed haplotype analysis within block 4 was not able to isolate the effect on WBC count to any one particular SNP or combination of SNPs (data not shown), suggesting that there may be more than one important SNP in this locus, or alternatively, that there is a causal SNP tagged by these three SNPs that we have not genotyped. Of the two sliding window peaks for *WRN*, one of them included *WRN* (rs2230009) and the other included *WRN* (rs2725362), located in block 2 and block 4, respectively (Figure 2). Haplotype analysis suggested that only these two SNPs were associated with a decreased WBC count (data not shown).

Deletion of the yeast DNA helicase BLM and WRN control factor SGS1 and growth sensitivity to benzene metabolite hydroquinone

Given the highly significant associations identified between one or more SNPs in *BLM* and *WRN* on the WBC count in benzene-exposed workers, and the fact that *BLM* and *WRN* belong to the same RecQ family of DNA helicases, which play a critical role in maintaining genome stability, we carried out experimental studies to evaluate the impact of loss of DNA helicase activity on cell growth in the presence of a key benzene toxic metabolite, hydroquinone. Yeast *BY4743* wild-type and *SGS1* deletion mutant were treated with the benzene metabolite, hydroquinone. Sgs1 is a yeast DNA helicase homologous in function to the BLM and WRN gene products. Therefore, deletion of *SGS1* in yeast is equivalent to deletion of *BLM* and *WRN* in humans. Exposure to increasing concentrations of hydroquinone resulted in a longer lag phase in the yeast wild-type with no apparent differences in growth rate in the exponential phase. In *sgs1*Δ, hydroquinone exposure adversely affected both the lag time and growth rate to a greater extent than in the wild-type, particularly at high doses (Figure 3).

Knockdown of WRN increases the susceptibility of human TK6 cells to hydroquinone toxicity

To further investigate the relationship between WRN and the cytotoxicity of hydroquinone, we generated stabilized human hematopoietic TK6 cells with depleted WRN expression using specific shRNA (shWRN) to knock down the gene (Figure 4A). Human TK6 cells treated with shWRN showed a statistically significant increase in sensitivity to hydroquinone treatment when compared with control TK6 cells treated with non-specific shRNA, especially at high concentrations of hydroquinone (Figure 4B).

Table III. Odds ratios and 95% confidence interval of having WBC <4000/μl blood in relation to SNPs in four genomic maintenance and DNA repair genes among benzene exposed subjects

Base pair position		WBC <4000	WBC ≥ 4000			WBC ≥ 5000			WBC ≥ 6000			WBC ≥ 7000		
		n	*n*	OR (95% CI)[a]	*P*[b]	*n*	OR (95% CI)[a]	*P*[b]	*n*	OR (95% CI)[a]	*P*[b]	*n*	OR (95% CI)[a]	*P*[b]
BLM	CC	6	96			69			40			16		
rs2270132	AC	15	109	2.5 (0.9–7.0)	0.08	67	4.2 (1.4–13)	**0.013**	33	5.7 (1.6–20)	**0.0083**	14	8.4 (1.5–48)	**0.018**
IVS19−499C>A	AA	9	35	5.7 (1.8–18)	**0.0033**	26	6.8 (1.9–25)	**0.0034**	10	13.9 (2.9–67)	**0.0011**	3	8.9 (1.2–67)	**0.035**
	AA+AC	24	144	3.2 (1.2–8.5)	**0.018**	93	5.0 (1.7–15)	**0.0034**	43	7.5 (2.2–25)	**0.0012**	17	8.6 (1.7–42)	**0.008**
Trend					**0.0031**			**0.0024**			**0.0007**			**0.018**
BLM	GG	19	190			136			73			30		
rs414634	GT	11	48	1.9 (0.8–4.4)	0.16	27	3.0 (1.1–7.8)	**0.029**	10	4.0 (1.2–13)	**0.027**	3	4.3 (0.8–24)	0.10
IVS21+1617A>C	TT		2			1			10	4.0 (1.2–13)	**0.027**	3	4.3 (0.8–24)	0.10
	TT+GT	11	50	1.8 (0.8–4.1)	0.20	28	2.9 (1.1–7.6)	**0.031**		4.0 (1.2–13)	**0.027**		4.3 (0.8–24)	0.10
Trend					0.26			**0.035**						
BLM	AA	13	140			97			55			21		
rs16944894	AG	11	81	2.2 (0.9–5.6)	0.10	53	2.5 (0.9–7.0)	0.07	21	5.0 (1.4–17)	**0.012**	11	4.2 (0.9–20)	0.08
4793 bp 3′ of ST	GG	3	16	5.2 (1.0–26)	**0.045**	12	5.0 (0.9–28)	0.07	7	7.2 (1.0–54)	0.05	1		
	GG+AG	14	97	2.5 (1.0–6.0)	**0.047**	65	2.8 (1.1–7.4)	**0.033**	28	5.3 (1.6–17)	**0.0054**	12	5.1 (1.1–24)	**0.039**
Trend					**0.023**			**0.023**			**0.0070**			**0.020**
RAD51	CC	21	213			147			77			31		
rs4924496	CT	9	27	4.0 (1.6–10)	**0.0041**	15	6.2 (2.0–19)	**0.0014**	6	6.8 (1.7–28)	**0.0075**	2	12.8 (1.3–130)	**0.031**
IVS3+1932T>C														
TP53	TT	13	136			96			54			20		
rs12951053	GT	11	81	1.4 (0.6–3.4)	0.43	53	2.0 (0.8–5.2)	0.16	23	2.3 (0.8–6.8)	0.14	12	1.2 (0.3–4.8)	0.81
IVS7+92T>G	GG	6	23	3.2 (1.0–10)	**0.050**	13	6.2 (1.6–24)	**0.0077**	6	10 (1.8–58)	**0.0080**	1	58 (1.9–1800)	**0.021**
	GG+GT	17	104	1.8 (0.8–3.9)	0.16	66	2.6 (1.1–6.3)	**0.032**	29	3.2 (1.2–8.8)	**0.023**	13	2.0 (0.6–7.1)	0.29
Trend					0.06			**0.0080**			**0.0065**			0.07
WDR79	CC	10	138			92			52			20		
rs2287499	CG	18	85	3.6 (1.5–8.8)	**0.0042**	59	4.0 (1.6–10)	**0.0042**	27	4.4 (1.5–13)	**0.0071**	12	4.0 (0.9–17)	0.06
Ex1−230C>G	GG	2	17	1.9 (0.4–9.9)	0.47	11	2.6 (0.4–16)	0.32	4	7 (0.6–80)	0.12	1	63 (0.8–5200)	0.07
	GG+CG	20	102	3.3 (1.4–7.8)	**0.0063**	70	3.8 (1.5–9.6)	**0.0048**	31	4.5 (1.6–13)	**0.0052**	13	4.4 (1.1–18)	**0.039**
Trend					**0.027**			**0.014**			**0.0059**			**0.022**

[a]OR, odds ratio; CI, confidence interval. ORs were adjusted for age, sex, current smoking, current alcohol drinking, BMI, recent infections, ln air benzene exposure, and ln air toluene exposure in the month before phlebotomy.
[b]*P* values <0.05 are bolded.

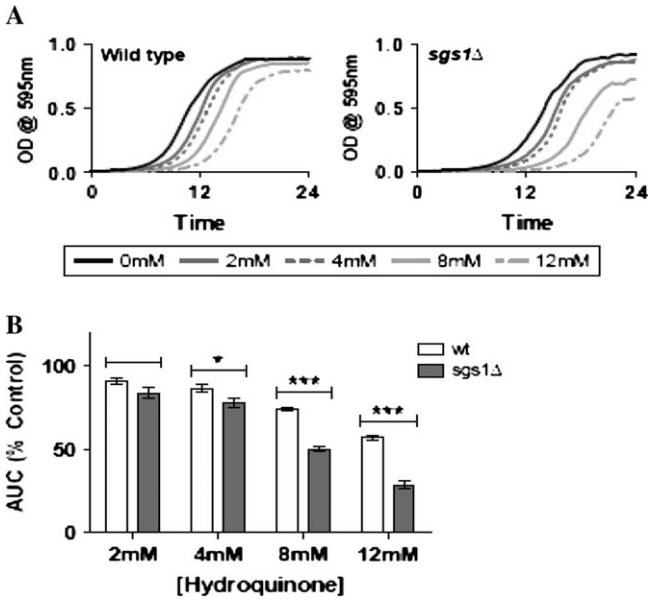

Fig. 3. (**A**) Growth curves for yeast BY4743 wild-type and *sgs1*Δ treated with of hydroquinone (HQ). Exposure to increasing concentrations of HQ resulted in a longer lag phase in the yeast wild-type with no apparent differences in growth rate in exponential phase. In *sgs1*Δ, HQ exposure adversely affected both the lag time and growth rate in a higher degree than in the wild-type, particularly at high doses. The growth curves represent averaged data from three technical replicates. Curves were smoothened and the error bars omitted for clarity purposes. The inhibitory concentration 20 of HQ for wild-type is 4 mM. (**B**) Total growth was quantified for wild-type and *sgs1*Δ by calculating the area under the growth curve (AUC). The bars represent the normalized AUC averages for wild-type and *sgs1*Δ in 2, 4, 8 and 12 mM HQ with standard errors. Except for 2 mM HQ, all other HQ treatments induced a decrease in the growth of *sgs1*Δ that were significantly different from corresponding treatments in the wild-type ($^{*}P < 0.05$; $^{***}P < 0.001$).

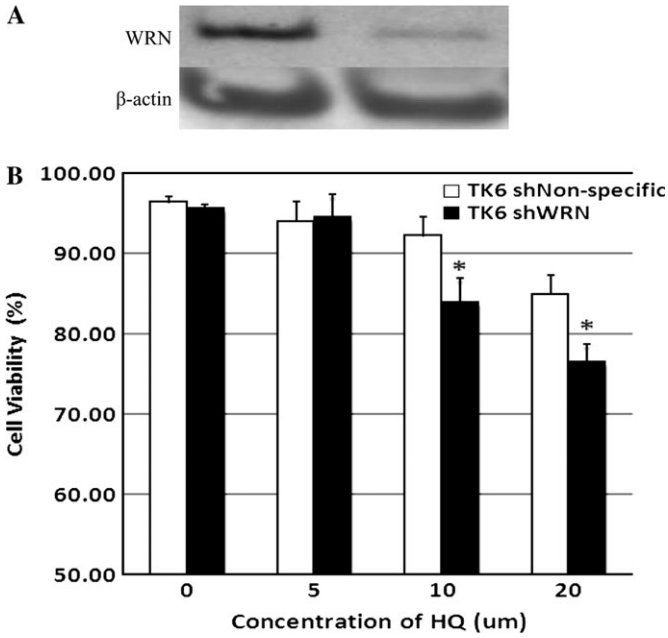

Fig. 4. (**A**) Knockdown of WRN in TK6 cells using specific shRNA. Whole-protein lysates were collected from TK6 cells following either shWRN- or shNon-specific transfection, and the protein levels of WRN were analyzed by western blotting. WRN expression was depleted by >90% when normalized against levels of β-actin, the loading control in the cells treated with shWRN. (**B**) TK6 cells with either shWRN or universial non-specific shRNA were treated with 5, 10 and 20 μm hydroquinone for 24 h. Total number of cells was counted using a hemocytometer with the trypan blue exclusion assay in unexposed cultures and in those treated with hydroquinone (HQ). The cells with shWRN showed the increased sensitivity to hydroquinone at 10 and 20 μm compared with the cells with universial non-specific shRNA control ($^{*}P < 0.05$).

Discussion

We conducted an analysis of the influence of 1395 SNPs drawn from 411 genes that are potentially involved in carcinogenic processes on peripheral WBC counts among 250 workers exposed to benzene and 140 unexposed controls and found that association signals clustered in genes (*BLM*, *TP53*, *RAD51*, *WDR79* and *WRN*) related to DNA repair and genomic maintenance. Further, *in vitro* functional studies provided evidence that the BLM and WRN gene products play a role in the toxic benzene metabolite hydroquinone-induced growth sensitivity. Given that *BLM*, *TP53*, *RAD51* and *WRN* all play a critical role in the homologous DNA recombination pathway, our findings suggest that this process is a key component of susceptibility to benzene-induced hematotoxicity in humans.

Cycling between two key benzene metabolites, quinone and hydroquinone, can generate free radicals that are able to form additional reactive oxygen species (6,23) and cause damage to protein, lipids and DNA. The damage to DNA can lead to double-strand breaks (24), chromosomal abnormalities and carcinogenesis if not properly repaired. Further, exposure to benzene metabolites has been shown to increase homologous recombination (HR) in mammalian (25,26) and yeast cells (24). Thus, DNA repair enzymes that maintain genomic stability after DNA damage may be crucial determinants of interindividual variability in response to benzene's toxic effects. It is therefore striking that our findings clustered in genes that play a role in repairing DNA and maintaining genomic stability. *BLM*, *WRN*, *RAD51* and *TP53* are all involved in the homologous DNA recombination pathway, which is required for genetic exchanges such as meiosis, repair of DNA and the segregation of chromosomes during cell division (27). BRCA2 also plays a role in HR, and it is noteworthy that we previously reported a modest association between *BCRA2* rs1801496 and benzene hematotoxicity, particularly for granulocytes, in this study population (4). This SNP was included on the Oligo Pool analyzed here, but its FDR value for association with the total WBC count was well >0.05 and it was not considered in subsequent analyses. Double-strand break repair through HR repair is one of the major DNA repair processes for genomic maintenance. *BLM* and *WRN* belong to the RecQ family of DNA helicases, which play a critical role in maintaining genome stability, including DNA replication, recombination, transcription and DNA repair. The RecQ family of DNA helicases is one of the most highly conserved group of DNA helicases across species. Humans produce five (BLM, WRN, RECQ1, 4 and 5) RecQ proteins. Loss of function of a RecQ family member at the cellular level leads to increased chromosomal aberrations (28), suggesting crucial roles for these proteins in maintaining large-scale genome stability.

Germ line mutations in *BLM* and *WRN* result in the rare autosomal recessive genomic instability disorders, i.e. Bloom syndrome and Werner syndrome (28,29). These genetic disorders are associated with genomic instability and cancer predisposition (28,29). BLM or WRN deficiencies are associated with an increase in chromosome aberration, sister chromatid exchanges (30) and replication abnormalities (31). BLM and WRN regulate HR through their ability to restrain by preventing inappropriate recombination (32). Mitotic recombination has been found to generate loss of heterozygosity in a variety of cancer types such as acute myeloid leukemias, breast cancer, bladder cancer and in gastrointestinal stromal tumors (33–36). Our findings suggest that a functioning HR system may be essential to protect from benzene-related hematotoxicity.

Galvan *et al.* (37) carried out an *in vitro* study and found that depletion in WRN results in a decrease in cell proliferation and an

enhanced susceptibility to cytotoxicity caused by hydroquinone, a toxic metabolite of benzene. Hydroquinone-treated WRN-depleted HeLa cells exhibited an increase in the amount of DNA double-strand breaks and an elevated DNA damage response suggesting that *WRN* plays a key role in benzene toxicity (37). In the present study, we show similar effects in human hematopoietic TK6 cells, where the knockdown of WRN using specific shRNA increased the susceptibility of TK6 cells to hydroquinone toxicity. Studies in yeast *in vitro* also reported here provide additional support for the requirement of functioning DNA helicases to protect from toxicity caused by hydroquinone. Further, our observation that a SNP in *TP53* also influenced benzene-induced hematotoxicity is consistent with the observation that TP53 can attenuate the ability of BLM and WRN to inhibit BLM and WRN helicase activities by binding to BLM and WRN helicases (27,38,39).

RAD51 is one of the most crucial components of HR repair because of its ability to catalyze the strand exchange between single-stranded and double-stranded DNA (40). *RAD51*-knockout mouse (RAD51$^{-/-}$) cells result in early embryonic lethality (41). Inactivation of RAD51 leads to chromosome breaks or aberrations, mutagenesis and cell death (42,43). Genetic studies have shown that *RAD51* 135G>C is associated with reduced risk of acute myeloid leukemia, breast cancer and ovarian cancer risk (44,45) and an increased risk of therapy-related acute myeloid leukemia (46). This variant is located in the 5′ untranslated region of the RAD51 messenger RNA and results from breast cancer patients suggest that the C allele may associate with increased level of the *RAD51* messenger RNA levels (47). We did not find a significant effect of this SNP. However, this SNP is in the same haplotype block with the two *RAD51* SNPs (rs11852786 and rs4924496) that we found to be associated with WBC count among benzene-exposed workers. Animal studies have shown that *RAD51* transcript levels were increased in male hematopoietic stem cells after exposure to the benzene metabolite 1,4-benzoquinone (48). A complementary DNA microarray study of p53-knockout mice using mouse bone marrow tissue has shown that RAD51 is markedly down-regulated after 2 weeks of benzene exposure (49).

In summary, our study provides strong support that interindividual variation in hematotoxicity caused by occupational benzene exposure is associated with genetic variation and in particular could be related to one or more genes that play a critical role in DNA repair and genomic maintenance. Further, our findings suggest that there are subgroups of individuals exposed to benzene below the current USA occupational standard who are particularly susceptible to benzene's toxic effects. These findings need to be replicated in other populations exposed to benzene, and additional analyses are needed to identify causal, functional variants. However, the clustering of some of the most highly significant findings in one pathway, and the interrelationship between the function of these genes provides further support for our observations.

Supplementary material

Supplementary Tables 1–5 can be found at http://carcin.oxfordjournals.org/

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