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Genetic Variants in Antioxidant Genes Are Associated With Diisocyanate-Induced Asthma

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Diisocyanates are a common cause of occupational asthma, but risk factors are not well defined. A case-control study was conducted to investigate whether genetic variants of antioxidant defense genes, glutathione S-transferases (*GSTM1*, *GSTT1*, *GSTM3*, *GSTP1*), manganese superoxide dismutase (*SOD2*), and microsomal epoxide hydrolase (*EPHX1*) are associated with increased susceptibility to diisocyanate-induced asthma (DA). The main study population consisted of 353 Caucasian French-Canadians from among a larger sample of 410 diisocyanate-exposed workers in three groups: workers with specific inhalation challenge (SIC) confirmed DA (DA^+, $n = 95$); symptomatic diisocyanate workers with a negative SIC (DA^-, $n = 116$); and asymptomatic exposed workers (AW, $n = 142$). Genotyping was performed on genomic DNA, using a 5′-nuclease PCR assay. The *SOD2* rs4880, *GSTP1* rs1695, and *EPHX1* rs2740171 variants were significantly associated with DA in both univariate and multivariate analyses. In the first logistic regression model comparing DA^+ and DA^- groups, *SOD2* rs4880, *GSTM1* (null), *GSTP1* rs762803, and *EPHX1* rs2854450 variants were associated with DA ($p = 0.004$, $p = 0.047$, $p = 0.021$, $p < 0.001$, respectively). Genotype combinations *GSTT1*GSTP1* rs762803, *GSTM1*EPHX1* rs2854450, *EPHX1* rs2740168EPHX1* rs1051741, and *GSTP1* rs762803**EPHX1* rs2740168 were also associated with DA in this model ($p = 0.027$, $p = 0.002$, $p = 0.045$, $p = 0.044$, respectively). The *GSTP1* rs1695 and *EPHX1* rs1051741 and rs2740171 variants showed an association with DA in the second model comparing DA^+ and AW groups ($p = 0.040$, $p = 0.019$, $p = 0.002$, respectively). The *GSTM3* rs110913**EPHX1* rs1051741 genotype combination was also associated with DA under this model ($p = 0.042$). The results suggest that variations in *SOD2*, *GST*, and *EPHX1* genes and their interactions contribute to DA susceptibility.**

Key Words: **diisocyanates; occupational asthma; antioxidant; genetics; single-nucleotide polymorphism.**

Occupational asthma (Mapp *et al.*, 2002) is characterized by variable airflow obstruction and/or airway hyperresponsiveness due to a particular exposure in the workplace and may account for 10–25% of all adult cases of asthma. Isocyanates, low molecular weight reactive chemicals used in diverse industrial processes, are one of the most frequently reported causes of OA in the workplace. Toluene diisocyanate (TDI), 4,4′-diphenylmethane diisocyanate (MDI), and hexamethylene diisocyanate (HDI) are the most commonly used monomers in industry and estimated to cause asthma in 5–15% of workers with long-term exposure (Bernstein, 1996; Mapp, 2001; Wisnewski and Redlich, 2001). The pathophysiology of diisocyanate-induced asthma (DA) involves chronic airway inflammation and oxidative stress in the lungs. Oxidative stress is caused by reactive oxygen and nitrogen species generated by activated inflammatory cells and bronchial epithelial cells resulting from tissue injury following inhalation of diisocyanates (Lantz *et al*., 2001; Lummus *et al*., 2011; Rahman *et al*., 2006). *In vivo* and *in vitro* studies have shown that diisocyanates alter thiol redox homeostasis of airway epithelial cells. In a mouse model, expression

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of genes involved in oxidative stress and thiol redox balance was increased following polymeric HDI exposure (Lee *et al*., 2005). Human serum albumin–conjugated TDI was found to induce oxidative stress in bronchial epithelial cells (Hur *et al*., 2009). Another study reported altered expression of proteins involved in oxidant/antioxidant–mediated airway inflammation in MDI-asthma patients (Kim *et al*., 2010). These findings suggest that oxidative stress is a major contributor to persistent airway inflammation and tissue damage in DA. An imbalance in redox systems favoring an oxidative environment has been associated with airflow limitation and airway remodeling in asthma (Comhair and Erzurum, 2010).

A number of enzymatic antioxidants, including glutathione S-transferases (GSTs), manganese superoxide dismutase (SOD2), and microsomal epoxide hydrolase (EPHX1) play a major protective role in redox imbalance in the lung as well as help regulate inflammatory responses (Bowler, 2004; Rahman *et al*., 2006). Because oxidative stress is an important early event in the pathway for diisocyanate-induced respiratory damage, genetic variability within genes coding for antioxidant defense systems can directly influence the ultimate expression of DA. The most commonly studied antioxidant enzymes include the GSTs (*GSTM1*, *GSTP1*, and *GSTT1*, chromosomes 1p13.3, 11q13, and 22q11.2, respectively). GSTs catalyze the conjugation of electrophilic compounds with reduced glutathione (GSH). *GSTP1* is the most highly expressed gene in the respiratory epithelium. The Ile105→Val105 substitution in *GSTP1* (rs1695) has been reported to alter the kinetics of the enzyme. Higher and lower activities of the Val105 allele were reported in conjugating different substrates and minor allele homozygosity was shown to confer a protective effect on asthma (Fryer *et al*., 2000; Johansson *et al*., 1998). *GSTM1* and *GSTT1* are both expressed weakly in the lung (Anttila *et al*., 1993; Cantlay *et al*., 1994). Homozygous deletion of *GSTM1* and *GSTT1* genes results in complete absence of enzyme activity and the null alleles have been shown to confer susceptibility to atopic bronchial and childhood asthma (Ivaschenko *et al*., 2002; Kabesch *et al*., 2004; Karam *et al*., 2012). Genetic association studies identified that *GSTM1* null genotype was associated with increased susceptibility for DA whereas workers with the *GSTP1* Val105Val were at reduced risk (Mapp *et al*., 2002; Piirilä *et al*., 2001).

SOD2 (chromosome 6q25.3) is an essential first line of defense against superoxide formed by the tetravalent reduction of oxygen during mitochondrial electron transport and catalyzes the dismutation of superoxide radicals into hydrogen peroxide. SOD2, one of the three known isoforms of SOD, is unique in that it is inducible by stimuli that cause oxidative stress (Hassan, 1988). The *SOD2* gene contains a single-nucleotide polymorphism (SNP) that results in an Ala→Val substitution (rs4880), which decreases the activity of SOD2. The rs4880 SNP has been widely studied and found to be associated with several respiratory diseases including bronchial hyperresponsiveness (BHR) and childhood asthma (Kuo Chou *et al*., 2010; Siedlinski *et al*., 2009).

EPHX1 (chromosome 1q42.1) is involved in the detoxification of epoxides and expressed in most tissues, including airway epithelium. Two common variations located in exon 3 (Tyr113His, rs1051740) and exon 4 (His139Arg, rs2234922) have been reported to reduce and increase *in vitro* enzyme activity, respectively (Hassett *et al*., 1994). These variants were extensively studied and found to be associated with asthma, emphysema, and chronic obstructive pulmonary disease (COPD) (DeMeo *et al*., 2007; Lakhdar *et al*., 2010; Tung *et al*., 2011; Xiao *et al*., 2004).

Based on the significance of antioxidant enzyme activities in asthma pathogenesis, the present candidate-gene association study was designed to investigate the role of *GST*, *SOD2*, and *EPHX1* polymorphisms as predictors of susceptibility to DA.

MATERIALS AND METHODS

Study participants. The main study population consisted of 353 Caucasian French-Canadians from among a larger sample of 410 diisocyanate (HDI, MDI, and TDI)-exposed workers. The main study analyses were conducted only on the Caucasian French-Canadians to avoid the possibility of bias due to population stratification (Heiman *et al*., 2004); supplemental analyses were also conducted on the entire sample, as described below. The population comprised of three distinct phenotypes including: (1) 95 workers diagnosed with DA (DA^+) based on a positive specific inhalation challenge (SIC) test; (2) 116 workers reporting respiratory symptoms at work in whom DA was excluded based on a negative SIC; and (3) 142 HDI-exposed asymptomatic worker controls (AWs). Symptomatic subjects were recruited from occupational pulmonary disease clinics located in Canada (Hôpital du Sacré-Coeur, Montreal; Hôpital Laval, Sainte-Foy; University Health Network, Toronto) and Spain (Fundación Jiménez Díaz, Madrid and Hospital Vall d'Hebron, Barcelona). The subjects underwent SIC with the appropriate work-relevant diisocyanate chemicals according to previously described protocols (Malo *et al*., 1999; Sastre *et al*., 2003). Patients were classified as DA^+ or DA^- based on their positive and negative responses to SIC, respectively. A decrease in FEV_1 of at least 20% from prechallenge baseline during the early and/or late asthmatic response was defined as a positive SIC test. AW controls were recruited in Quebec, Canada from HDI-exposed painters. Data regarding age, sex, ethnicity, smoking status, time of exposure, and respiratory symptoms were collected by questionnaire. Atopy was evaluated by skin prick testing to common aeroallergens, defined by a positive reaction of at least 3 mm greater than saline control for at least one allergen. Whole blood was collected for genetic testing. All subjects gave informed consent, and the study protocol is approved and renewed annually by institutional review boards of National Institute for Occupational Safety and Health and each participating institution.

Gene selection and genotyping. Genomic DNA was extracted from whole-blood samples using the QIAamp blood kit (QIAGEN Inc., Chatsworth, CA). Genotyping was performed on genomic DNA using a 5′-nuclease PCR assay. Primers and probes were designed using the TaqMan Assay-by-Design service from Applied Biosystems (Foster City, CA). Candidate genes were selected based on their functional role in oxidative stress and inflammation. The QuickSNP version 1.1 was used to select 14 tagSNPs within the candidate genes that had a minor allele frequency (MAF) > 5% and an $r^2 > 0.8$ in Caucasians (Grover *et al*., 2007). In addition, deletion of *GSTM1* and *GSTT1* genes were analyzed. PCR amplification was performed in a volume of 25 µl containing 10 ng genomic DNA, 12.5 µl 2× TaqMan Universal Master Mix, 200 nM of probe and 900 nM of primer. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 92°C for 30 s and 60°C for 1 min. Amplification was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems). Positive and negative controls were used within each run of PCR amplification. All samples with ambiguous results were repeated

as were a random selection of 10% of all samples to ensure laboratory quality control. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was used as internal control for the deletion polymorphisms of *GSTT1* and *GSTM1* (Johnson, 2006; Johnson *et al*., 2004). Sequences for primers and probes are available on request.

Statistical analyses. The analysis was restricted to Caucasian French-Canadians in order to minimize bias due to population stratification. Also, the numbers of subjects recruited from other non-Caucasian French-Canadian ($n = 31$) and Spanish ($n = 26$) populations were too small to independently support statistical modeling. However, identical statistical analyses were also conducted on the entire sample and results of those are included as Supplementary data. Potential associations between each SNP and DA were tested using chi-square tests for single SNP associations. The sample size did not support the inclusion of all possible pairs of two-way interactions in a logistic regression model; therefore, interactions were screened using Breslow-Day tests for homogeneity of odds ratios. Those pairs of SNPs having nonhomogeneous odds ratios for disease ($p < 0.05$) were entered as two-way interactions into a logistic regression model using backward selection (also at $p < 0.05$), adjusting for demographic variables that retained significance when included in the model (age, smoking status, and type of diisocyanate exposure [HDI vs. MDI or TDI] or exposure duration). In the first model, comparison of DA$^+$ and DA$^-$ symptomatic groups was conducted whereas DA$^+$ and AW controls were included in the second model. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

Subject Characteristics

The demographic characteristics of the study groups included in the statistical analyses are described in Table 1. All participants in the statistical analyses were Caucasian French-Canadians. Mean age was higher in the DA$^+$ and DA$^-$ groups than AW controls (42.4, 40.1 vs. 30.4 years; $p < 0.001$). Diisocyanate exposure differed significantly between the groups (overall $p < 0.001$). Although the duration of work exposure was similar between the DA$^+$ and DA$^-$ worker groups (144.6 vs. 165.8 months, $p = 0.278$), AW controls had less exposure to isocyanates than both groups (65.8 months, $p < 0.001$). The frequency of atopy was similar in all three groups (overall $p = 0.899$). The prevalence of smoking was significantly different between DA$^+$ and AW controls ($p < 0.001$). The overall type and the severity of the respiratory symptoms (e.g., cough, wheezing, shortness of breath, tightness in chest) were similar in symptomatic groups. For example, the frequency of wheezing, cough, and shortness of breath in DA$^+$ versus DA$^-$ groups were, respectively, 111 (46.8 %) versus 114 (48.1%), 111 (42.4%) versus 128 (48.8%), and 121 (44.3%) versus 138 (50.6%). However, the frequency of symptoms was significantly different between DA$^-$ and AW groups. The frequency of wheezing, cough, and shortness of breath in DA$^-$ versus AWs were 114 (48.1%) versus 12 (5.1%), 128 (48.8%) versus 23 (8.8%), and 138 (50.6%) vs. 14 (5.1%), respectively. The allele frequencies in the control population were similar to those determined in other studies involving Caucasian populations and were in Hardy-Weinberg equilibrium (data not shown). The demographic characteristics of the initial study sample are given in Supplementary table 1.

Genotype Distribution

Table 2 shows the distribution of genotypes in the study population and the p-values represent the comparison of the proportions of genotypes between two groups. The rs4880 (SOD2), rs2740171 (EPHX1), and rs1695 (GSTP1) were the only candidate SNPs that were individually significantly associated with the DA diagnosis. The distribution of the SOD2 rs4880 genotype was significantly different in DA$^+$ workers (27.4% homozygous major allele, 36.8% heterozygous, 35.8% homozygous minor allele) compared to DA$^-$ workers (30.2% homozygous major allele, 49.1% heterozygous, 20.7% homozygous minor allele, p=0.043) and AW controls (24.1% homozygous major allele, 54.6% heterozygous, 21.3% homozygous minor allele, p=0.015). The distribution of the EPHX1 rs2740171 SNP was also significantly different among DA$^+$ cases (49.5% homozygous major allele, 46.3% heterozygous, 4.2% homozygous minor allele) compared to AW controls (65.5% homozygous major allele, 31.0% heterozygous, 3.5% homozygous minor allele) (p=0.046), as was the distribution of GSTP1 rs1695 among DA$^+$ cases (49.5% homozygous major allele, 41.1% heterozygous, 9.5% homozygous minor allele) compared to AW controls (33.8% homozygous major allele, 56.3% heterozygous, 9.9% homozygous minor allele) (p = 0.046).

TABLE 1
The Demographic Characteristics of the Study Sample

	DA$^+$	DA$^-$	AWs	DA$^+$ vs. DA$^-$	DA$^+$ vs. AWs	Overall *p* Values
N (French-Canadians)	95	116	142			
Sex, M/F	84/11	105/11	132/10	0.620	0.228	0.483
Age at diagnosis ± s.e.	42.4 ± 1.2	40.1 ± 0.9	30.4 ± 0.6	0.152	**<0.001**	**<0.001**
Diisocyanate exposure (HDI/MDI/TDI)	53/22/20	92/19/5	142/0/0	**<0.001**	**<0.001**	**<0.001**
Duration of exposure, months ± s.e.	144.6 ± 14.4	165.8 ± 13.0	65.8 ± 2.3	0.278	**<0.001**	**<0.001**
Height, cm ± s.e.	171.5 ± 0.8	172.4 ± 0.8	175.6 ± 0.7	0.424	**<0.001**	**<0.001**
Atopy (positive/negative)	56/36	63/46	76/54	0.659	0.719	0.899
Smoker (current/ex/never)	16/36/43	39/37/38	52/27/63	**0.0159**	**<0.001**	**0.001**
Pack-years ± s.e.	11.9 ± 1.6	11.3 ± 1.3	5.8 ± 0.7	0.775	**<0.001**	**<0.001**

TABLE 2
Distribution of Genotype Frequencies Between the Groups

	DA$^+$ (n = 95)	DA$^-$ (n = 116)	AWs (n = 142)	χ^2 or Fisher's exact (p values)	
Gene/SNP ID	N (%)	N (%)	N (%)	DA$^+$ vs. DA$^-$	DA$^+$ vs. AWs
GSTP1 rs1695				0.554	**0.046**
1.1	47 (49.5)	51 (44.0)	48 (33.8)		
1.2	39 (41.1)	49 (42.2)	80 (56.3)		
2.2	9 (9.5)	16 (13.8)	14 (9.9)		
GSTP1 rs762803				0.292	0.069
1.1	39 (41.1)	36 (31.0)	41 (28.9)		
1.2	38 (40.0)	57 (49.1)	78 (54.9)		
2.2	18 (18.9)	23 (19.8)	23 (16.2)		
GSTT1				0.078	0.094
Null	23 (24.0)	17 (14.7)	22 (15.5)		
Present	72 (75.8)	99 (88.3)	120 (84.5)		
GSTM1				0.766	0.352
Null	47 (49.5)	55 (47.4)	79 (55.6)		
Present	48 (50.5)	61 (52.6)	63 (44.4)		
GSTM3 rs1109138				0.970	0.827
1.1	30 (31.6)	36 (31.0)	48 (33.8)		
1.2	46 (48.4)	58 (50.0)	63 (44.4)		
2.2	19 (20.0)	22 (19.0)	31 (21.8)		
GSTM3 rs1537234				0.219	0.522
1.1	45 (47.4)	42 (36.2)	58 (40.9)		
1.2	34 (35.8)	54 (46.6)	61 (43.0)		
2.2	16 (16.8)	20 (17.2)	23 (16.2)		
GSTM3 rs1571858				0.987	0.743
1.1	51 (53.7)	61 (52.6)	69 (48.6)		
1.2	36 (37.9)	45 (38.8)	60 (42.3)		
2.2	8 (8.4)	10 (8.6)	13 (9.2)		
SOD rs4880				**0.043**	**0.015**
1.1	26 (27.4)	35 (30.2)	34 (24.1)		
1.2	35 (36.8)	57 (49.1)	77 (54.6)		
2.2	34 (35.8)	24 (20.7)	30 (21.3)		
EPHX1 rs1051740				0.213	0.085
1.1	53 (55.8)	59 (50.9)	71 (50.0)		
1.2	27 (28.4)	45 (38.8)	58 (40.8)		
2.2	15 (15.8)	12 (10.3)	13 (9.2)		
EPHX1 rs2234922				0.958	0.739
1.1	69 (72.6)	85 (73.9)	98 (69.0)		
1.2	23 (24.2)	26 (22.6)	37 (26.1)		
2.2	3 (3.2)	4 (3.5)	7 (4.9)		
EPHX1 rs1051741				0.565	0.466
1.1	80 (84.2)	100 (86.2)	112 (78.9)		
1.2	15 (15.8)	15 (12.9)	29 (20.4)		
2.2	0 (0.0)	1 (0.9)	1 (0.7)		
EPHX1 rs2260863				0.598	0.584
1.1	43 (45.3)	51 (44.4)	74 (52.1)		
1.2	41 (43.2)	55 (47.8)	54 (38.0)		
2.2	11 (11.6)	9 (7.8)	14 (9.9)		
EPHX1 rs2740168				0.655	0.153
1.1	48 (50.5)	52 (44.8)	55 (38.7)		
1.2	34 (35.8)	44 (37.9)	68 (47.9)		
2.2	13 (13.7)	20 (17.2)	19 (13.4)		
EPHX1 rs2740171				0.293	**0.046**
1.1	47 (49.5)	58 (50.0)	93 (65.5)		
1.2	44 (46.3)	47 (40.5)	44 (31.0)		
2.2	4 (4.2)	11 (9.5)	5 (3.5)		
EPHX1 rs2854450				0.652	0.550
1.1	65 (68.4)	73 (62.9)	88 (62.0)		
1.2	28 (29.5)	39 (33.6)	49 (34.5)		
2.2	2 (2.1)	4 (3.5)	5 (3.5)		
EPHX1 rs10915884				0.838	0.499
1.1	66 (69.5)	85 (73.3)	94 (66.2)		
1.2	26 (27.4)	28 (24.1)	46 (32.4)		
2.2	3 (3.2)	3 (2.6)	2 (1.4)		

Note. Genotypes, 1.1: homozygous for the major allele; 2.2: homozygous for the minor allele; 1.2 heterozygous. Bold values indicate statistical significance.

TABLE 3
Logistic Regression Model for Significant Variations, DA⁺ Versus DA− Groups

Model term	Estimate, β	S.E.	χ^2	OR (95% CI)	p value[a]
Intercept	0.5762	0.4565	1.59		0.207
SOD2 (rs4880)	0.4965	0.1706	8.47	2.70 (1.38, 5.27)	**0.004**
GSTT1	0.1373	0.3246	0.18	1.32 (0.37, 4.70)	0.672
GSTM1	–0.3736	0.1884	3.73	0.47 (0.23, 0.99)	**0.047**
EPHX1 (rs1091588)	0.1428	0.1968	0.53	1.33 (0.62, 2.88)	0.468
GSTP1 (rs762803)	0.9037	0.3925	5.30	6.10 (1.31, 28.4)	**0.021**
EPHX1 (rs2854450)	–0.8415	0.2456	11.7	0.19 (0.07, 0.49)	**<0.001**
EPHX1 (rs2740168)	0.0863	0.2687	0.10	1.19 (0.41, 3.41)	0.748
EPHX1 (rs1051741)	–0.6720	0.4799	1.396	0.26 (0.04, 1.71)	0.161
EPHX1 (rs2740171)	0.1777	0.1604	1.23	1.43 (0.76, 2.68)	0.268
*GSTT1***GSTP1* (rs762803)	–0.9071	0.4091	4.92	0.16 (0.03, 0.81)	**0.027**
*GSTM1***EPHX1* (rs2854450)	0.9970	0.3269	9.30	7.34 (2.04, 26.5)	**0.002**
EPHX1 (rs2740168)**EPHX1* (rs1051741)	1.0729	0.5361	4.00	8.55 (1.05, 69.9)	**0.045**
GSTP1 (rs762803)**EPHX1* (rs2740168)	–0.6212	0.3085	4.05	0.29 (0.09, 0.97)	**0.044**

[a]Adjusted for smoking status and type of exposure (HDI vs. MDI or TDI).

TABLE 4
Logistic Regression Model for Significant Variations in HDI-exposed Workers: DA⁺ Versus AW Controls

Model term	Estimate, β	S.E.	χ^2	OR (95% CI)	p value[a]
Intercept	–8.0666	1.4054	32.9		<0.0001
GSTP1 (rs1695)	–0.5646	0.2745	4.23	0.32 (0.11, 0.95)	**0.040**
EPHX1 (rs1051741)	1.1688	0.4981	5.51	10.36 (1.47, 72.96)	**0.019**
EPHX1 (rs2740171)	0.9140	0.2955	9.57	6.22 (1.95, 19.82)	**0.002**
GSTM3 (rs110913)	0.5129	0.3739	1.88	2.79 (0.64, 12.08)	0.170
GSTM3 (rs1109138)**EPHX1* (rs1051741)	–1.4045	0.6898	4.15	0.06 (0.01, 0.90)	**0.042**

[a]Adjusted for age, smoking status and duration of exposure.

Genetic Models

Tables 3 and 4 present the results of logistic regression models examining statistically significant SNPs and interactions associated with DA after adjusting for significant confounders (age, smoking status, type or duration of exposure). SNPs were dichotomized as carriers of the minor allele (homozygote or heterozygote) versus major allele homozygotes, except for SOD2 where homozygous carriers of the minor allele were compared to homozygote and heterozygote carriers of the major allele (Lummus *et al*., 2010). GSTT1 and GSTM1 deletions were dichotomized as present versus null. For the interaction terms, the odds ratio represents the odds of DA⁺ for carriers of at least one minor allele at both SNPs versus the odds of DA⁺ for any other genotype combination. The results of analyses on the larger sample that included subjects from Spain and non-Caucasian French-Canadians were similar to those in Tables 2–4, suggesting that population stratification was not a major problem (Supplementary data and Tables 2–4).

DA⁺ Versus DA⁻ Workers

The first model included DA⁺ and DA⁻ groups and adjusted the results for smoking status and type of diisocyanate exposure (HDI vs. MDI or TDI; Table 3). The homozygous minor allele of *SOD2* rs4880 SNP was associated with a higher risk of DA with an OR of 2.70 (95% confidence interval [CI]: 1.38–5.27). *GSTP1* rs762803 SNP also showed an association with DA under this model (OR: 6.10, 95% CI: 1.31–28.4). On the other hand, *GSTM1* (null) and the minor allele of *EPHX1* rs2854450 SNP conferred protection against DA (OR: 0.47, 95% CI: 0.23–0.99 and OR: 0.19, 95% CI: 0.07–0.49, respectively). Some SNPs conferred risk only in the presence of another SNP. The copresence of minor alleles of *GSTP1* rs762803**GSTT1* and *GSTP1* rs762803**EPHX1* rs2740168 was associated with a lower risk of DA (OR: 0.16, 95% CI: 0.03–0.81 and OR: 0.29, 95% CI: 0.09–0.97, respectively). On the other hand, the carriage of the GSTM1 deletion and the minor allele for *EPHX1* rs2854450 conferred an increased risk of disease with an OR of 7.34 (95% CI: 2.04–26.5).

HDI-Exposed DA⁺ Workers Versus HDI-Exposed AW Controls

The second logistic regression model included DA⁺ and AW groups and adjusted the results for age, smoking status and exposure months (Table 4). Only HDI-induced DA⁺ cases were taken into consideration because controls were exposed only to HDI. Although the *GSTP1* rs1695 minor alleles were significantly associated with protection against DA (OR: 0.32,

95% CI: 0.11–0.95), carriage of the minor alleles for *EPHX1* rs1051741 and for *EPHX1* rs2740171 conferred an increased risk for DA (OR: 10.36; 95% CI: 1.47–72.96 and OR: 6.22, 95% CI: 1.95–19.82, respectively). The copresence of minor alleles of *GSTM3* rs110913 and *EPHX1* rs1051741 was associated with a decreased risk of DA (OR: 0.06, 95% CI: 0.01–0.90).

DISCUSSION

This study showed significant associations between the *SOD2* rs4880, *EPHX1* 2740171, and *GSTP1* rs1695 variants and DA in both univariate and multivariate analysis. Furthermore, polymorphisms in *GSTM1*, *GSTT1*, *GSTP1*, *EPHX1*, and *GSTM3* genes showed significant associations with DA after adjustment for confounding variables, either individually or in combination.

Previous studies showed that reduced SOD2 activity in the lung is related to airway inflammation and BHR in asthmatic patients, confirming the role of SOD2 in airway homeostasis (Comhair *et al*., 2005; Smith *et al*., 1997). Homozygosity for the minor allele of the *SOD2* rs2842958 SNP was found to be a risk factor for BHR in a general population (Siedlinski *et al*., 2009), and a recent study showed a statistically significant association between the minor allele of SNP rs4880 and an increased risk of asthma in children (Kuo Chou *et al*., 2010). Two other studies, on the other hand, failed to show any association between rs4880 genotypes and asthma (Holla *et al*., 2006; Kinnula *et al*., 2004). Our results showed that the homozygous variant genotype of SNP rs4880 may confer an increased risk for DA. Mitochondria are a major source of relative oxygen species in cells and the rs4880 SNP occurs within the mitochondrial targeting sequence of *SOD2* gene (McLennan and Degli Esposti, 2000; Shimoda-Matsubayashi *et al*., 1996). *In vitro* studies have shown that expression of the Val allele results in decreased mitochondrial import and mRNA stability of SOD2 and influences efficient neutralization of intracellular superoxide radicals (Sutton *et al*., 2003, 2005). Although the rs4880 SNP has been found to be associated with several oxidative stress-related diseases, there is no report on its influence on DA risk. To our knowledge, this is the first report showing a statistically significant association of the rs4880 variant allele with DA and supports the hypothesis that less-efficient defense against oxidative stress products in mitochondria may contribute to the pathogenesis of DA.

GSH is one of the major antioxidant defenses at the air-lung interface. GSH is used as an essential cofactor by GSTs in conjugation with electrophilic substances. GSTP1 is the most strongly expressed GST in the respiratory epithelium. The most commonly studied GSTP1 Val105 (rs1695) variant has been reported to be both protective and a risk factor for asthma (Aynacioglu *et al*., 2004; Salam *et al*., 2007; Spiteri *et al*., 2000; Tamer *et al*., 2004). Functional studies showed that the Val105 allele alters the active site of the enzyme and results in altered substrate-specific conjugation activity (Johansson *et al*., 1998). However, the reports on the role of this variant in DA are inconsistent. Mapp *et al*. (2002) reported a protective effect of the Val105 allele against TDI-asthma whereas Piirilä *et al*. (2001) did not find such effect in workers exposed to TDI, HDI, and MDI. Consistent with Mapp *et al*. (2002), our results showed a reduced risk for DA in workers with the Val105 allele in comparison to AWs. The other SNP (rs762803) in the *GSTP1* gene showed a significant association with increased risk of DA when compared with DA^- symptomatic workers. The functional consequence of this SNP is not known. Therefore, it is not possible to sort out whether this SNP is itself functional or exerts its effect through linkage disequilibrium with a neighboring functional SNP.

Piirilä *et al*. (2001) also examined variations of the *GSTM1*, *GSTM3*, and *GSTT1* genes in workers with DA and found the *GSTM1* null genotype to be associated with a 1.89-fold increased risk of TDI-asthma. In our population, *GSTM1* null genotype was associated with a reduced risk of DA in the first logistic regression model. Although the *GSTT1* null genotype and *GSTM3* rs1109138 showed no association with DA individually in both models, they conferred protection against DA in combination with the *GSTP1* rs762803 (Model 1) and *EPHX1* rs1051741 (Model 2), respectively (Tables 3 and 4). These observations suggest that the absence or presence of a significant single-locus main effect does not exclude the possibility of important interactions between the loci at an integrated physiological level. It is likely that gene-gene interactions modify the expression of DA.

The role of EPHX1 in DA pathogenesis has not been previously investigated. Although *in vitro* studies showed a functional role for widely studied SNPs at exons 3 and 4, only modest effects on EPHX1 enzyme activity have been found in *in vivo* studies (Hosagrahara *et al*., 2004). In the present study, no association of these variants with DA was found. However, MAF distribution of the *EPHX1* rs2740171 was significantly different between DA^+ subjects and AW controls in univariate analysis. This variant allele also conferred an increased risk for DA with OR 6.22 after adjusting for confounders (Table 4). The *EPHX1* SNP rs2854450 showed a protective effect against DA in the first model. *EPHX1* SNPs also interacted with variations within *GST* genes. The combination of the *GSTM1* (null) and *EPHX1* rs2854450 variant allele was associated with an increased DA risk, whereas the minor alleles of *EPHX1* rs2740168 and *GSTP1* rs762803 interacted to reduce risk of DA. The latter finding is consistent with an interactive role of *GSTP1* and *EPHX1* variants reported in a study of childhood asthma (Salam *et al*., 2007). Although the functional significance of these SNPs is not known and it is not known whether any functional polymorphism is in linkage disequilibrium with them, we speculate that individuals with multiple risk alleles across *EPHX1* and *GST* genes are more susceptible to diisocyanate exposure, and that this sensitivity may contribute to the development of DA. It is also possible that associations seen may be mediated by interactions with other polymorphic loci.

The major strengths of this study include a well-defined phenotype, and examination of candidate genes based on their functional role in disease pathogenesis. In addition to comparing with exposed workers without any evidence of respiratory disease, we were able to incorporate a worker group representing respiratory symptoms that are not caused by diisocyanate exposure. We were also able to test our genetic associations while adjusting for potential independent confounding factors such as atopy, smoking history, exposure duration, and specific diisocyanate exposure. The major limitations include small sample size due to rarity of DA and the issue of multiple interferences. Also, small numbers of subjects carrying specific alleles or genotype combinations resulted in large confidence intervals. Another limitation is that the AW controls were younger and had less exposure to diisocyanates than cases. This was unintentional due to difficulty in the recruitment of age-matched workplace controls and may be problematic in terms of detection of age-related associations. The results were not corrected for multiple comparisons because our analyses were based on well-defined roles of the selected genes in disease process. Instead, we reported all tests that reached the 0.05 level of significance. We believe that the disadvantage of small sample size was minimized by well-characterized phenotype and hypothesis-driven analytic strategy.

Taken together, this case-control study reports that the *SOD2* rs4880, *EPHX1* 2740171, and *GSTP1* rs1695 variants are significantly associated with DA supporting the hypothesis that genetic variability within antioxidant defense systems contributes to the pathogenesis of this disease. After adjustment for confounding variables, variants of *GSTM1*, *GSTT1*, *GSTP1*, *EPHX1*, and *GSTM3* genes also showed significant positive or negative associations with DA. This may suggest that the effect of these variations can be detected after the variability of the outcome is reduced by adjusting for the other associated factors and in the context of gene-gene, gene-exposure interactions. Although there are inconsistencies in the literature, some of the markers included in this study have been investigated in different asthma phenotypes and replicated in different populations (Vercelli, 2008; Weiss *et al*., 2009). For example, *GSTM1* null and *GSTP1* Ile105Val genotypes were found to be associated with increased susceptibility to diesel exhaust particles and ozone exposure (Gilliland, 2009; Romieu *et al*., 2006). In addition, the SOD2 Ala allele was reported to be associated with childhood asthma (Kuo Chou *et al*., 2010). Our results support the involvement of these variations in asthma, particularly in asthma phenotypes driven by oxidant stress-dependent pulmonary inflammation. Further studies are warranted to confirm these findings in an independent replication cohort and to characterize functional role of these markers in disease process.

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SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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