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RESEARCH ARTICLE

Genetic variants in *TNFα*, *TGFB1*, *PTGS1* and *PTGS2* genes are associated with diisocyanate-induced asthma

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Abstract

Diisocyanates are the most common cause of occupational asthma, but risk factors are not well defined. A case-control study was conducted to investigate whether genetic variants in inflammatory response genes (*TNFα*, *IL1α*, *IL1β*, *IL1RN*, *IL10*, *TGFB1*, *ADAM33*, *ALOX-5*, *PTGS1*, *PTGS2* and *NAG-1/GDF15*) are associated with increased susceptibility to diisocyanate asthma (DA). These genes were selected based on their role in asthmatic inflammatory processes and previously reported associations with asthma phenotypes. The main study population consisted of 237 Caucasian French Canadians from among a larger sample of 280 diisocyanate-exposed workers in two groups: workers with specific inhalation challenge (SIC) confirmed DA (DA^+, $n = 95$) and asymptomatic exposed workers (AW, $n = 142$). Genotyping was performed on genomic DNA, using a 5′ nuclease PCR assay. After adjusting for potentially confounding variables of age, smoking status and duration of exposure, the *PTGS1* rs5788 and *TGFB1* rs1800469 single nucleotide polymorphisms (SNP) showed a protective effect under a dominant model (OR = 0.38; 95% CI = 0.17, 0.89 and OR = 0.38; 95% CI = 0.18, 0.74, respectively) while the *TNFα* rs1800629 SNP was associated with an increased risk of DA (OR = 2.08; 95% CI = 1.03, 4.17). Additionally, the *PTGS2* rs20417 variant showed an association with increased risk of DA in a recessive genetic model (OR = 6.40; 95% CI = 1.06, 38.75). These results suggest that genetic variations in *TNFα*, *TGFB1*, *PTGS1* and *PTGS2* genes contribute to DA susceptibility.

Keywords

Cytokine, diisocyanates, inflammation, occupational asthma, single nucleotide polymorphism (SNP)

History

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Introduction

Diisocyanates, low-molecular weight reactive chemicals used in diverse industrial processes, are one of the most frequently reported causes of occupational asthma in the work-place. 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; Wisnewski & Redlich, 2001). Diisocyanates bind selectively to human proteins and these protein conjugates act as antigens that induce airway inflammation with the release of inflammatory mediators. Of these mediators, cytokines play a critical role in asthma pathogenesis by orchestrating the allergic inflammatory response (Maestrelli et al., 1995). Elevated levels of both T-helper (T_H)-1 and T_H2 cytokines have been demonstrated in the airways of subjects with different asthma phenotypes, supporting the heterogenic nature of disease (Broide et al., 1992; Fisseler-Eckhoff et al., 2011; Maestrelli et al., 1995, 1997; Mapp et al., 2009; Piirila et al., 2008). Animal and *in vitro* models revealed multiple roles of cytokines in non-specific inflammatory processes as well as specific immune events in response to diisocyanate exposure (Chiung et al., 2010; Matheson et al., 2002; Swierczynska-Machura et al., 2012).

Although many studies investigated the association between single nucleotide polymorphisms (SNP) in pro-inflammatory cytokine genes and asthma phenotypes (Che et al., 2014; Padron-Morales et al., 2013; Panek et al., 2014; Savenije et al., 2011; Zeyrek et al., 2008), there is only one study that focused on DA. Beghé et al. (2004) studied tumor necrosis factor-α (*TNFα*) A-308G polymorphism in TDI-induced asthma but found no

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significant difference in frequency of this variant between cases and asymptomatic exposed subjects. The interleukin-1 (*IL1*), *IL10*, *TNF*α and transforming growth factor-β (*TGFB1*) genes have not previously been studied in relation to DA susceptibility.

Other genes focused on in this study included *ADAM33* (ADAM metallopeptidase domain 33), *PTGS1* (prostaglandin-endoperoxide synthase-1, previously *COX1*), *PTGS2* (previously *COX2*), *ALOX5* (arachidonate 5-lipoxygenase) and *NAG-1/GDF15* (non-steroidal anti-inflammatory drug-activated gene). *ADAM33* is the first reported asthma-susceptibility gene identified by positional cloning (van Eerdewegh et al., 2002). It is highly expressed in epithelium and airway smooth muscle cells and plays an important role in airway remodeling (Tripathi et al., 2014). It has been suggested that altered ADAM33 protein function may promote inflammation through impaired or enhanced release of cytokines and growth factors (Cakebread et al., 2004). Cyclo-oxygenases are key enzymes that catalyze the biosynthesis of prostaglandins, which are essential mediators of inflammation in asthma (Dubois et al., 1998). ALOX5 is the first enzyme in the biosynthetic pathway leading to the production of leukotrienes and plays an important role in allergic and inflammatory diseases (Duroudier et al., 2009; Radmark et al., 2007). NAG-1/GDF15 is a member of the TGFβ cytokine superfamily that plays an important role in controlling inflammatory responses by reducing leukocyte recruitment and maintaining homeostasis (Zarbock & Rossaint, 2011). Numerous studies showed associations between genetic variants of *PTGS1*, *PTGS2*, *ADAM33* and *ALOX5* genes and allergic asthma, but these variations have not been studied for association with DA (Holloway et al., 2008; Shi et al., 2005, 2008; Song et al., 2013). In addition, the *NAG1/GDF15* variants were not explored in asthma phenotypes.

Since the impact of a given gene on the phenotype is influenced by occupational and environmental exposures, gene–environment interaction explains phenotype variance better than two factors considered separately. Occupational asthma (OA) is an excellent model for studying gene–environment interactions since the causal agent can be identified with specific inhalation challenge (SIC) and the lag period between initial exposure and onset of sensitization and clinical symptoms can be followed. Therefore, it is important to study asthma candidate genes in OA since different occupational and environmental exposures might have a different effect on the phenotype. Based on their role in asthma pathogenesis and previously reported associations with asthma phenotypes, the present candidate gene association study was designed to investigate the role of SNP in a group of inflammatory response genes as predictors of susceptibility to DA. The identification of variations in specific genes involved in the DA process might lead to a better understanding of the underlying disease pathways and, thus, guide for the development of more effective therapeutic and preventive strategies.

Materials and methods

Study participants

The main study population consisted of 237 Caucasian French Canadians from among a larger sample of 280 diisocyanate (HDI, MDI and TDI)-exposed workers. The main study analyses were conducted on only the Caucasian French Canadians to avoid the possibility of bias due to population stratification (Heiman et al., 2004). Also, the numbers of subjects recruited from other non-Caucasian-French Canadian and Spanish populations were too small to independently support statistical modeling. However, identical statistical analyses were also conducted on the entire sample and the results are presented as supplementary data. Of the 237 participants, 95 workers were diagnosed with DA (DA^+) based on a positive specific inhalation challenge (SIC) test, while 142 HDI-exposed asymptomatic workers (AW) served as controls. We did not perform a separate analysis on HDI-induced DA^+ cases and controls since the majority of DA^+ subjects were exposed to HDI and overall numbers would be too small for meaningful statistical analysis.

Symptomatic subjects were recruited from occupational pulmonary disease clinics located in Canada (Sacre Coeur Hospital, Montreal; Laval Hospital, Sainte-Foy; University Health Network, Toronto) and Spain (Fundacion Jimenez Diaz, 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). A decrease in FEV_1 of at least 20% from pre-challenge 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 the National Institute for Occupational Safety and Health and each participating institution.

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 Assay-by-Design™ service from Applied Biosystems (Foster City, CA). PCR amplification was performed in a volume of 25 µl containing 10 ng genomic DNA, 12.5 µl 2X Taqman® Universal Master Mix, 200 nM probe and 900 nM 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.

Statistical analyses

Comparisons between groups on demographic variables were analyzed using chi-square tests or analysis of variance for discrete and continuous variables, respectively. Initial analyses, including tests for Hardy–Weinberg Equilibrium (HWE), allele and genotype frequencies were calculated using the Fisher's Exact Test. Unadjusted odds ratios (OR) were calculated using contingency tables. Adjusted ORs were calculated using logistic regression while adjusting for age, exposure time (months) and smoking status (current/ex/never). Sex and atopy were not significant in any models and were not included in the final analysis. Since underlying genetic models are unknown *a priori*, the association between each SNP and DA status was analyzed using three genetic models. These include a dominant model (comparing homozygous wild-type genotype with variant allele-carrying genotypes), a recessive model (comparing wild-type allele-carrying genotypes with homozygous variant genotype) and an additive model. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC). SNAP2 tools were used to update annotations of significant SNP according to dbSNP135 and to find proxy SNP within 500 kb based on linkage disequilibrium (LD) and physical distance (Johnson et al., 2008). Since SNP

located in a genomic region with various transcription factor (TF) binding sites are more likely to influence transcriptional regulation, we searched functional elements overlapping with the selected SNP. RegulomeDB was used to annotate SNP with known and predicted regulatory elements (Boyle et al., 2012).

Results

Subject characteristics

The demographic characteristics of the study groups included in the statistical analyses are described in Table 1. The participants included in this analysis were Caucasian French-Canadians. Mean age was higher in the DA^+ group than AW controls (42.4 versus 30.4 years). While the DA^+ group consisted of subjects exposed to HDI, MDI and TDI (53:22:20), AW controls were exposed only to HDI in the workplace. AW controls had less duration of exposure to diisocyanates than the DA^+ group (65.8 versus 144.6 months). The prevalence of smoking was significantly different between the DA^+ group and AW controls ($p < 0.0001$). The frequency of atopy was similar between the two groups (58.9% in DA^+, 53.5% in AW controls). Allele frequencies in the control population were similar to those determined in other studies involving Caucasian populations and were in HWE (data not shown). Demographic characteristics of the initial study sample are shown in Supplementary Table 1.

Genotype distribution and genetic models

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 frequency of the *PTGS2* rs20417 SNP showed only a marginal significance between the DA^+ group and AW controls ($p = 0.057$) in the univariate analysis.

Table 3 presents the results of the logistic regression model after adjusting for potentially confounding variables of age, smoking status and duration of exposure. In a dominant model, the *PTGS1* rs5788 and *TGFB1* rs1800469 SNP were associated with a protection against DA (OR = 0.38; 95% confidence interval (CI) = 0.17, 0.89, $p = 0.026$ and OR = 0.38; 95% CI = 0.18, 0.74, $p = 0.005$) while the *TNF*α rs1800629 SNP conferred an increased risk of disease (OR = 2.08; 95% CI = 1.03, 4.17, $p = 0.041$). On the other hand, the *PTGS2* rs20417 SNP showed an association with an increased risk of DA in a recessive model (OR = 6.40; 95% CI = 1.06, 38.75, $p = 0.043$). This SNP also showed a trend towards significance in an additive model (OR = 5.99; 95% CI = 0.98, 36.57, $p = 0.053$). Results of analyses on the larger sample that included subjects from Spain and non-Caucasian French Canadians were similar to those in Tables 2 and 3, suggesting population stratification did not bias the findings (Supplementary Tables 2 and 3).

Regulatory information for significant associations

The four significant SNP identified from data analysis were used as inputs to the SNAP SNP Annotation and Proxy Search tools to update SNP IDs according to dbSNP135 and to find additional SNP in complete LD ($r^2 = 1$). This led to the identification of an additional 17 correlated SNP using data from the International HapMap Project (International HapMap Consortium et al., 2010). The total set of 21 SNP was then used as input to the RegulomeDB web resource, which integrates data from the ENCODE projects and other data sources regarding various types of functional assays including DNaseI-seq, ChIP-seq, RNAseq and eQTL analyses (Boyle et al., 2012). RegulomeDB scores of all the significant and correlated SNPs are presented in Supplementary Table 4. Two SNPs (*TGFB1* rs1800469 and *TNF*α rs1800629) were likely to affect binding and linked to expression of a gene target (MGC4093 and AIF, respectively) (RegulomeDB scores = 1b and 1f, respectively). The *PTGS2* rs20417 SNP was also likely to affect binding with a RegulomeDB score of 2b. There were no regulatory data available for the *PTGS1* rs5788 SNP. Table 4 shows the SNP with RegulomeDB scores between 1–3 (scoring refers to available data types supporting a functional role for the variant). All SNP with putative regulatory function (RegulomeDB Score 1–6) are given in Supplementary Table 5.

Discussion

This is the first comprehensive study to explore potential associations of genetic variants in inflammatory genes with DA. The allele frequencies of 19 SNP in 11 genes were determined in a Caucasian worker population of European background. Two SNP in *TNF*α and *TGFB1* genes were significantly associated with DA. TNFα is one of the major pro-inflammatory cytokines located within the human major histocompatibility complex (MHC). This region has been repeatedly linked to asthma (Daniels et al., 1996; Xu et al., 2001). Previous studies demonstrated increased levels of TNFα in asthmatic airways and reported an association of *TNF*α rs1800629 (A-308G) SNP with asthma phenotypes as well as elevated total serum IgE levels (Ricciardolo et al., 2013; Sharma et al., 2006; Shin et al., 2004; Witte et al., 2002). A recent meta-analysis involving 34 studies confirmed these findings and reported rs1800629 SNP as a risk factor for asthma (Yang et al., 2014). The -308A allele has been associated with increased expression levels of TNFα in stimulated human white blood cells (Wilson et al., 1997). In our analysis, carriage of the -308A allele was associated with an increased risk of DA.

Beghè et al. (2004) investigated this SNP in TDI-induced asthma, but did not find any association. The difference between two study results could be related to differences in population genetic structure and type of diisocyanate exposure. We found

Table 1. Demographics of study groups.

Variable	DA^+	AW	DA+ versus AWs (*p* value)
n	95	142	
Sex (M/F)	84/11	132/10	0.2499
Age (years, SEM)	42.4, 1.24	30.4, 0.63	<0.0001
Exposure (HDI/MDI/TDI)	53/22/20	142/0/0	<0.0001
Exposure Duration (months, SEM)	144.6, 14.4	65.8, 2.3	<0.0001
Atopy (Yes/No)	56/36	76/54	0.7819
Smoking (Current/Ex/Never)	16/36/43	52/27/63	<0.0001

DA^+, exposed workers with SIC confirmed diisocyanate asthma; AW, asymptomatic diisocyanate exposed controls.

Table 2. Distribution of genotype frequencies between the groups.

Gene/SNP ID*	DA+ ($n=95$) n (%)	AWs ($n=142$) n (%)	Fisher's exact p values
ADAM33 S1 (rs3918396)			
1.1	75 (80.6)	122 (85.9)	0.3661
1.2	13 (14.0)	17 (12.0)	
2.2	5 (5.4)	3 (2.1)	
ADAM33 ST + 5 (rs597980)			
1.1	23 (24.5)	41 (28.9)	0.5444
1.2	50 (53.2)	65 (45.8)	
2.2	21 (22.3)	36 (25.3)	
ADAM33 T1 (rs2280091)			
1.1	70 (74.5)	113 (80.1)	0.1681
1.2	22 (23.4)	28 (19.9)	
2.2	2 (2.1)	0 (0.00)	
ALOX5 (G-1699A) (rs4986832)			
1.1	59 (64.1)	95 (66.9)	0.7180
1.2	28 (30.4)	42 (29.6)	
2.2	5 (5.4)	5 (3.5)	
PTGS1 (C644A) (rs5788)			
1.1	69 (73.4)	102 (72.3)	0.7237
1.2	20 (21.3)	34 (24.1)	
2.2	5 (5.3)	5 (3.6)	
PTGS2 (G-765C) (rs20417)			
1.1	65 (69.1)	91 (64.5)	0.0566
1.2	23 (24.5)	48 (34.0)	
2.2	6 (6.4)	2 (1.4)	
PTGS2 (G3050C) (rs5277)			
1.1	68 (72.3)	105 (74.5)	0.3547
1.2	26 (27.2)	33 (23.4)	
2.2	0 (0.00)	3 (2.1)	
NAG1/GDF15 (H6D) (rs1058587)			
1.1	49 (52.1)	76 (53.9)	0.8561
1.2	41 (43.6)	57 (40.4)	
2.2	4 (4.3)	8 (5.7)	
NAG1/GDF15 (V9L) (rs1059519)			
1.1	41 (43.6)	56 (39.4)	0.6283
1.2	41 (43.6)	71 (50.0)	
2.2	12 (12.8)	15 (10.6)	
IL10 (G-1082A) (rs1800896)			
1.1	27 (29.0)	39 (27.5)	0.3051
1.2	45 (48.4)	81 (57.0)	
2.2	21 (22.6)	22 (15.5)	
IL10 (C-819T) (rs1800871)			
1.1	56 (60.2)	72 (51.1)	0.4177
1.2	31 (33.3)	57 (40.4)	
2.2	6 (6.5)	12 (8.5)	
IL1α (G + 4845T) (rs17561)			
1.1	45 (48.4)	78 (54.9)	0.5935
1.2	38 (40.9)	51 (35.9)	
2.2	10 (10.7)	13 (9.2)	
IL1β (C + 3953T) (rs1143634)			
1.1	48 (51.6)	88 (62.0)	0.0635
1.2	43 (46.2)	46 (32.4)	
2.2	2 (2.2)	8 (5.6)	
IL1β (C-511T) (rs16944)			
1.1	38 (40.9)	63 (44.4)	0.8735
1.2	40 (43.0)	58 (40.8)	
2.2	15 (16.1)	21 (14.8)	
IL1 RN (T + 2018C) (rs419598)			
1.1	50 (53.2)	75 (52.8)	1.0000
1.2	37 (39.4)	56 (39.4)	
2.2	7 (7.4)	11 (7.8)	
TGFB (C-509T) (rs1800469)			
1.1	53 (57.0)	63 (44.4)	0.1693
1.2	30 (32.3)	61 (43.0)	
2.2	10 (10.7)	18 (12.7)	
TGFB codon 10 (T/C) (rs1982073)			
1.1	34 (39.1)	55 (39.0)	0.4746
1.2	33 (37.9)	62 (44.0)	
2.2	20 (23.0)	24 (17.0)	
TNFα (G-238A) (rs361525)			
1.1	86 (92.5)	129 (90.9)	0.7369
1.2	7 (7.5)	11 (7.7)	
2.2	0 (0.0)	2 (1.4)	
TNFα (G-308A) (rs1800629)			
1.1	56 (60.2)	104 (73.2)	0.0996
1.2	33 (35.5)	35 (24.7)	
2.2	4 (4.3)	3 (2.1)	

*1.1, homozygous for the major allele; 2.2, homozygous for the minor allele; 1.2, heterozygous.

that the rs1800629 SNP (RegulomeDB score, 1f) is an eQTL (expression quantitative trait loci) for *AIF1* gene (Allograft Inflammatory Factor 1) and is situated in the Sp1 (specificity protein 1) binding motif. *AIF1* is also located in the MHC region and is induced by cytokines. It is suspected that *AIF1* is involved in the regulation of vascular smooth muscle cell growth. Sp1 is a ubiquitously expressed transcriptional activator protein that binds GC-rich regions of DNA to regulate gene transcription (Suske, 1999; Tan & Khachigian, 2009). Sp1 has been shown to be involved in the regulation of VEGF transcription that is a critical mediator of asthma pathogenesis. Clifford et al. (2012) suggested that increased Sp1 binding might contribute to elevated VEGF secretion in asthmatics. Sp1 was also found to be one of the transcription factors involved in the regulation of basal and inducible 5-lipoxygenase gene transcriptions (Silverman et al., 1998). It is possible that the *TNFα* rs1800629 SNP alters Sp1 binding to this region and influences downstream biological processes.

The other significantly associated SNP (rs1800469, -509 C/T) is located in the promoter region of the *TGFB1* gene. TGFB1 is an immunoregulatory cytokine that is increased in the airways of asthmatic individuals (Ohno et al., 1996). The rs1800469 is located within a YY1 (Ying Yang 1) consensus binding site and is known to regulate transcriptional activity (Silverman et al., 2004). The rs1800469 SNP has been associated with elevated gene expression and circulating TGFB1 and IgE levels (Grainger et al., 1999; Jacob et al., 2013). Increased transcriptional activity of the T allele, as compared to the C allele, has been reported (Grainger et al., 1999). While some studies showed an association between variant rs1800469 and allergic asthma (Li et al., 2007; Pulleyn et al., 2001; Sharma et al., 2009; Silverman et al., 2004; Wu et al., 2010; Yang et al., 2011), others reported no association with asthma or other allergies (Buckova et al., 2001; Hobbs et al., 1998). In our analysis, carriage of the T allele was associated with a protection against DA. TGFB1 is a potent suppressor of inflammation and found to be protective in acute animal models of asthma (Alcorn et al., 2007; Li et al., 2006; Nakao et al., 2000). On the other hand, excess TGFB1 activity was reported to induce fibrosis, pulmonary inflammation and structural remodeling (Crosby & Waters, 2010; McMillan et al., 2005). This is the first report showing an association between *TGFB1* rs1800469 variant and DA. Our findings are consistent with the immunomodulatory role of *TGFB1* in the asthmatic process and suggest that the same SNP could be protective or adverse depending on environmental and other genetic factors.

In line with previous observations, RegulomeDB showed evidence of regulatory function for the rs1800469 SNP (score, 1b). RegulomeDB cites rs1800469 is an eQTL for *MGC4093*, affects binding of POLR2A, CREBBP, CTCF and falls within the COUPTF binding motif. *MGC4093* is one of the two genes (the other is *MGC2055*) with unknown function in the 30 kb region surrounding the *TGFB1* gene. This gene lies between

Table 3. Logistic regression model for significant variations: DA^+ versus AW controls.

Genetic model	Additive		Dominant		Recessive	
Gene/SNP	OR (95% CI)	*p* Value†	OR (95% CI)	*p* Value†	OR (95% CI)	*p* Value*
ADAM33/rs3918396	0.57 (0.08, 4.05)	0.572	0.50 (0.19, 1.30)	0.155	0.63 (0.09, 4.46)	0.645
ADAM33/rs597980	0.88 (0.33, 2.35)	0.799	1.35 (0.63, 2.89)	0.448	0.64 (0.28, 1.44)	0.279
ADAM33/rs2280091	1.63 (0.75, 3.53)†	0.216	1.81 (0.85, 3.87)	0.124	N/A	
ALOX5/rs4986832	1.81 (0.30, 10.98)	0.517	1.06 (0.52, 2.18)	0.874	1.82 (0.31, 10.81)	0.512
PTGS1/rs5788	0.54 (0.10, 3.02)	0.479	0.38 (0.17, 0.89)	0.026	0.71 (0.13, 3.77)	0.688
PTGS2/rs20417	5.99 (0.98, 36.57)	0.053	0.99 (0.50, 1.97)	0.969	6.40 (1.06, 38.75)	0.043
PTGS2/rs5277	1.14 (0.54, 2.37)†	0.734	1.02 (0.49, 2.10)	0.963	N/A	
GDF15/rs1058587	0.42 (0.06, 2.91)	0.379	0.97 (0.50, 1.86)	0.923	0.41 (0.06, 2.79)	0.365
GDF15/rs1059519	0.94 (0.31, 2.81)	0.907	0.93 (0.48, 1.81)	0.830	0.98 (0.35, 2.74)	0.962
IL10/rs1800896	1.71 (0.67, 4.37)	0.264	0.95 (0.47, 1.93)	0.882	2.04 (0.90, 4.60)	0.087
IL10/rs1800871	0.32 (0.07, 1.40)	0.131	0.58 (0.30, 1.12)	0.105	0.39 (0.09, 1.63)	0.196
IL1α/rs17561	1.47 (0.52, 4.18)	0.470	1.33 (0.69, 2.53)	0.396	1.32 (0.49, 3.60)	0.584
IL1β/rs1143634	0.40 (0.07, 2.33)	0.310	1.34 (0.69, 2.57)	0.387	0.34 (0.06, 1.90)	0.219
IL1β/rs16944	0.75 (0.28, 1.98)	0.557	1.02 (0.53, 1.97)	0.949	0.70 (0.28, 1.74)	0.440
IL1RN/rs419598	0.84 (0.24, 3.01)	0.792	0.96 (0.50, 1.84)	0.901	0.85 (0.24, 2.94)	0.795
TGFB/rs1800469	0.41 (0.14, 1.19)	0.101	0.38 (0.18, 0.74)	0.005	0.64 (0.23, 1.74)	0.380
TGFB/rs1982073	0.77 (0.30, 1.96)	0.581	0.61 (0.30, 1.23)	0.167	1.05 (0.45, 2.44)	0.915
TNFα/rs361525	0.81 (0.25, 2.59)†	0.723	0.59 (0.19, 1.84)	0.364	N/A	
TNFα/rs1800629	0.43 (0.05, 4.03)	0.456	2.08 (1.03, 4.17)	0.041	0.34 (0.04, 3.12)	0.341

*Adjusted for smoking status, duration of exposure and age.
†Marked SNP, heterozygotes versus major genotype; others, major variant versus minor variant; N/A, no minor genotype.

Table 4. Significant and correlated SNPs with putative regulatory function (RegulomeDB Score 1–3).

Gene	RegulomeDB score	Significant and correlated SNPs	Distance	eQTL	Protein binding	Binding motif
TGFB1	1b	rs1800469*	0	MGC4093	POLR2A, CREBBP, CTCF	COUPTF
	1f	rs1982072	1378	MGC4093		
	2b	rs2241712	3410		NFKB1, CDX2, USF1, SP1, EGR1, ZBTB7A, IRF1, POLR2A, ELF1, GAPBA, SPI1, MAX, NRF1, TBP	SREBP, SREBP1
TNFα	1f	rs1800629*	0	AIF1		SP1
PTGS2	2b	rs20417*	0		TFAP2A, TFAP2C	STAT1, E2F-1:DP-1, E2F-1:DP-2, E2F-4:DP-2, EWSR1-FLI1
PTGS1	2b	rs10306141	2085		CTCF, TRIM28, SMC3, RAD21	POU1F1

eQTL, expression quantitative trait loci; *Significant study SNPs.

TGFB1 rs1800469 and rs7045 and its transcripts have been reported in various UniGene libraries of lung tissue. The genomic region including *TGFB1* and *MGC4093* is organized in two large haplotype blocks in Caucasians. Based on association and LD patterns and the fact that only *TGFB1* spans both of these haplotype blocks, the *MGC4093* has been ruled out as a modifier of other lungs diseases such as chronic obstructive pulmonary disease and cystic fibrosis. *TGFB1* SNPs were proposed as a likely modifier of these diseases (Celedon et al., 2004; Drumm et al., 2005). Although these findings need to be replicated in an independent sample, our results are consistent with previous observations and suggest *TGFB1* rs1800469 SNP as a likely modifier of DA.

One of the proteins affected by this SNP is CTCF (the transcription regulator CCCTC-binding factor). It is a DNA insulator protein with the ability to block enhancers from activating their target genes via chromatin remodeling. CTCF was found to be essential for the GATA3/SATB1-mediated regulation of the T_H2 cytokine gene expression (Ribeiro de Almeida et al., 2009). Other affected protein CREBBP (CREB binding protein) is one of the transcriptional co-activators with intrinsic histone acetyltransferase (HAT) activity. HAT and histone deacetylase (HDAC) regulate chromatin acetylation and likely play a key role in induction of inflammatory genes. Several studies reported an imbalance between activities of HATs and HDAC in bronchial biopsies, alveolar macrophages and peripheral blood mononuclear cells in asthma (Cosio et al., 2004; Ito et al., 2002). POLR2A (RNA II Polypeptide A) is a DNA-dependent RNA polymerase catalyzing transcription of DNA into RNA. The rs1800469 variant may play an important role in affecting the binding of these regulatory proteins and consequently the risk of DA. The same SNP is also situated in the COUP binding motif. COUPTFs (COUP transcription factor) are transcription factors that play key roles in the development and function of the immune system (Leid et al., 2004). It is possible that the overlap between rs1800469 and this binding motif influence the optimal expression of particular inflammatory genes. Examining these interactions in the context of DA pathogenesis could yield additional insight into the molecular mechanisms involved.

The *PTGS2* rs20417 is located within the promoter region and the -765C allele has been shown to have significantly lower promoter activity *in vitro* compared with the -765G allele (Papafili et al., 2002). PTGS2 is highly inducible by mitogenic and pro-inflammatory stimuli and involved in the regulation of inflammatory responses. Increased expression of *PTGS2* is observed in the airway epithelium and submucosa of asthmatics

(Sousa et al., 1997; Taha et al., 2000). While some studies reported associations of *PTGS2* SNPs with asthma phenotypes (Chan et al., 2007; Shi et al., 2008; Szczeklik et al., 2004), others found no relationship (Shi et al., 2004). In our analysis, the *PTGS2* rs20417 SNP conferred an increased risk of DA with an OR of 6.4 and showed evidence of regulatory function (RegulomeDB score, 2b). The rs20417 SNP has indications for binding TFAP2A (transcription factor AP-2 alpha), TFAP2C (Transcription Factor AP-2 Gamma) proteins. However, results of this interaction in the asthmatic process are unknown. This SNP is also located in the binding motifs for STAT1, E2F and EWSR1-FLI1. STAT (Signal transducer and activator of transcription) transcription factors play critical roles in regulating cell growth and differentiation, homeostasis and the immune response (Horvath, 2000; Ramana et al., 2000). E2F transcription factors are involved in the regulation of cell cycle and DNA replication in mammalian cells (Cam & Dynlacht, 2003). EWSR1 (EWS RNA-Binding Protein 1) acts as a transcriptional activator and plays a role in the initiation of transcription (Bertolotti et al., 1998). The overlap of the rs20417 SNP with these motif sites may have an impact on the regulation of gene expression.

The *PTGS1* rs5788 SNP showed a protective effect under a dominant model. *PTGS1* SNPs have been previously studied, but no association with asthma phenotypes (allergic and aspirin-intolerant asthma) was found (Shi et al., 2005). This is the first study to report an association between the *PTGS1* rs5788 SNP and an asthma phenotype. No regulatory information was found for that specific SNP.

The major strengths of this study include a well-defined phenotype and examination of candidate genes based on their functional role in disease pathogenesis. We were also able to test our genetic associations while adjusting for potential independent confounding factors such as age, smoking history and exposure duration. The major limitations include small sample size due to rarity of DA, lack of information regarding the relative exposure levels of the AW and DA groups and the issue of multiple interferences. 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. However, the AW group had mean exposure duration of 5.5 years. It has been reported that nearly 40% and 60% of subjects exposed to isocyanates become symptomatic within, respectively, 1 year and after 5 years of exposure (Malo et al., 1992). Therefore, it is reasonable to expect this exposure period to be sufficient for developing DA in susceptible individuals. The results were not corrected for multiple comparisons since our analyses were based on *a priori* hypotheses. Instead, we reported all tests that reached the 0.05 level of significance.

In conclusion, this case-control study reports, for the first time, an association between DA and SNPs in *TNFα*, *FB1*, *PTGS2* and *PTGS1* genes. Further studies are warranted to confirm these findings in an independent replication cohort and to characterize the functional role of these markers in DA susceptibility.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online
Supplementary Tables 1–5