Genetic variants with gene regulatory effects are associated with diisocyanate-induced asthma

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Background: Isocyanates are major causes of occupational asthma, but susceptibility and mechanisms of diisocyanate-induced asthma (DA) remain uncertain.
Objective: The aim of this study was to identify DA-associated functional genetic variants through next-generation sequencing (NGS), bioinformatics, and functional assays.
Methods: NGS was performed in 91 workers with DA. Fourteen loci with known DA-associated single nucleotide polymorphisms (SNPs) were sequenced and compared with data from 238 unexposed subjects. Ranking of DA-associated SNPs based on their likelihood to affect gene regulatory mechanisms in the lung yielded 21 prioritized SNPs. Risk and nonrisk oligonucleotides were tested for binding of nuclear extracts from A549, BEAS-2B, and IMR-90 lung cell lines by using electrophoretic mobility shift assays. DNA constructs were cloned into a pGL3 promoter vector for luciferase gene reporter assays.
Results: NGS detected 130 risk variants associated with DA (3.1×10^{-6} to 6.21×10^{-4}), 129 of which were located in noncoding regions. The 21 SNPs prioritized by using functional genomic data sets were in or proximal to 5 genes: cadherin 17 (*CDH17*; n = 10), activating transcription factor 3 (*ATF3*; n = 7), family with sequence similarity, member A (*FAM71A*; n = 2), tachykinin receptor 1 (*TACR1*; n = 1), and zinc finger and BTB domain-containing protein 16 (*ZBTB16*; n = 1). Electrophoretic mobility shift assays detected allele-dependent nuclear protein binding in A549 cells for 8 of 21 variants. In the luciferase assay 4 of the 21 SNPs exhibited allele-dependent changes in gene expression. DNA affinity precipitation and mass spectroscopy of rs147978008 revealed allele-dependent binding of H1 histones, which was confirmed by using Western blotting.
Conclusions: We identified 5 DA-associated potential regulatory SNPs. Four variants exhibited effects on gene regulation (*ATF* rs11571537, *CDH17* rs2446824 and rs2513789, and *TACR1* rs2287231). A fifth variant (*FAM71A* rs147978008) showed nonrisk allele preferential binding to H1 histones. These results demonstrate that many DA-associated genetic variants likely act by modulating gene regulation. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *Asthma, bioinformatics, diisocyanate, electrophoretic mobility shift assay, functional genomics, gene regulation, genetic, histone, isocyanate, luciferase assay, next-generation sequencing, occupational asthma, oligonucleotide, single nucleotide polymorphism*

Occupational asthma (OA) is a common occupational lung disorder.[1] Diisocyanate exposure accounts for approximately 25% of approved workers' compensation claims for OA.[2] Causative diisocyanate chemicals include hexamethylene diisocyanate,

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Supported by National Institute for Occupational Safety and Health (NIOSH)/Centers for Disease Control and Prevention (CDC) grant OH008795 (PI: D. I. Bernstein); a National Institutes of Health (NIH) shared instrumentation grant (S10 RR027015-01; PI: K. D. Greis); NIH grant P30 AR070549 for Cincinnati Children's Hospital Medical Center Functional Genomics Core; Cincinnati Children's Hospital Medical Center CpG Award 53553 (PI: M. T. Weirauch); a Lupus Research Institute "Novel Approaches" Award (PI: M. T. Weirauch); and NIH grant R21 HG008186 (PI: M. T. Weirauch).

Disclosure of potential conflict of interest: D. I. Bernstein has received research support from National Institute for Occupational Safety and Health (NIOSH)/Centers for Disease Control and Prevention (CDC) and consultancy fees from the International Isocyanate Institute outside the submitted work. A. Cartier received personal fees and other from AZ Pharma Canada, TEVA Pharma Canada, Sanofi Genzyme Canada, Boehringer Ingelheim Pharma Canada, GlaxoSmithKline Pharma Canada, and IC-EBM Canada outside the submitted work. C. Lemiere received grants from Institut de Recherche Robert Sauvé en santé et sécurité au Travail and grants from the Canadian Institutes of Health Research outside the submitted work. S. Tarlo has received reimbursement from the NIOSH/CDC for this study and support from grants during 2003-2018 (Workplace Safety & Insurance Board, Network of Centres of Research Expertise "Centre of Research Expertise in Occupational Disease [CRE-OD]" and a Verma Grant outside the submitted work and has a patent and book editor royalties with royalties paid. J. Sastre received payment from a National Institutes of Health grant and personal fees from Sanofi, Novartis, AstraZeneca, ALK-Abelló, and LETI outside the submitted work. L. P. Boulet has received payments for participation in multicenter research studies from AIM Therapeutics, Amgen, Asmacure, AstraZeneca, Axikin, GlaxoSmithKline, Hoffman La Roche, Novartis, Ono Pharma, Sanofi, and Takeda; research support from AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck, and Takeda; consultancy fees from AstraZeneca, Novartis, and Methapharm; royalties for coauthorship of UpToDate (occupational asthma); grants for production of educational materials from AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck Frosst, and Novartis; and conference fees from AstraZeneca, GlaxoSmithKline, Merck, Novartis, and Takeda. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 12, 2018; revised June 1, 2018; accepted for publication June 8, 2018.

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0091-6749/$36.00

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https://doi.org/10.1016/j.jaci.2018.06.022

Abbreviations used
ATF3: Activating transcription factor 3
CDH17: Cadherin 17
CEBPB: CCAAT/enhancer binding protein B
ChIP-Seq: Chromatin immunoprecipitation sequencing
DA: Diisocyanate-induced asthma
DAPA: DNA affinity purification assay
EMSA: Electrophoretic mobility shift assay
FAM71A: Family with sequence similarity 71, member A
GWAS: Genome-wide association study
MAF: Minor allele frequency
Nano-LC-MS/MS: Nano-liquid chromatography coupled to tandem mass spectrometry
NGS: Next-generation sequencing
OA: Occupational asthma
SIC: Specific inhalation challenge
SNP: Single nucleotide polymorphism
TACR1: Tachykinin receptor 1/Neurokinin 1 receptor
ZBTB16: Zinc finger and BTB domain-containing protein 16

methylene diphenyl diisocyanate, and toluene diisocyanate, which are used for manufacturing polyurethane foam products, metal surface paints, and other applications.[3,4] These chemicals remain important respiratory sensitizers and among the 10 most common agents associated with work-related asthma in the United States.[5,6]

Despite clinical resemblance to allergic asthma, allergic mechanisms for diisocyanate-induced asthma (DA) have not been identified, including biomarkers of sensitization (eg, specific IgE).[7,8] Thus an unmet need exists for markers that facilitate identification of workers with high DA susceptibility and early recognition of DA,[9,10] thereby preventing disability and persistent asthma.[11,12]

Genetic association studies of diisocyanate-exposed workers, including 2 genome-wide association studies (GWASs), have identified DA-associated genetic variants from multiple loci, some reaching genome-wide significance.[13-21] A potential study limitation is the small number of workers with DA able to be recruited (a relatively rare disorder). In the latter publications[19,20] a very well-defined disease phenotype of OA confirmed by using controlled specific inhalation challenge (SIC) testing with the work-relevant isocyanate chemical could explain the large number of reported disease-associated single nucleotide polymorphisms (SNPs). DA-associated SNPs have been found in genes encoding antioxidant enzymes,[13,21] HLA class I and II molecules,[18] T_H1/T_H2 cytokines and *CD14*,[14,16] and epithelial junctional proteins.[22] Previously, we replicated DA associations with 2 *CTNNA3* (α-T catenin) SNPs previously reported in a GWAS of Korean workers.[17,19] We also conducted a GWAS in 74 white workers with confirmed DA, leading to identification of 11 SNPs in 6 genetic loci that increase risk for DA ($P < 10^{-7}$) and 38 SNPs in 7 genetic loci with suggestive significance ($P < 10^{-6}$).[20]

The aim of this study was to further characterize loci of interest from our GWAS[20] and to identify functional risk alleles associated with confirmed DA. We used a targeted custom next-generation sequencing (NGS) approach to sequence the 14 genetic loci that had been identified in our previous studies.[17,20] Confirmed genetic risk variants were then prioritized based on their potential for altering gene regulatory mechanisms in relevant cell types. Prioritized DA-associated oligonucleotides were tested for binding of nuclear extract proteins by using electrophoretic mobility shift assays (EMSAs),[23] and genotype-dependent DNA regulatory activity was investigated in gene reporter assays.[24] Finally, using the DNA affinity purification assay (DAPA) and mass spectroscopy, we identified specific proteins bound to DA-associated genetic risk variants. Results from this study provide insight into the specific molecular mechanisms affected by DA-associated variants.

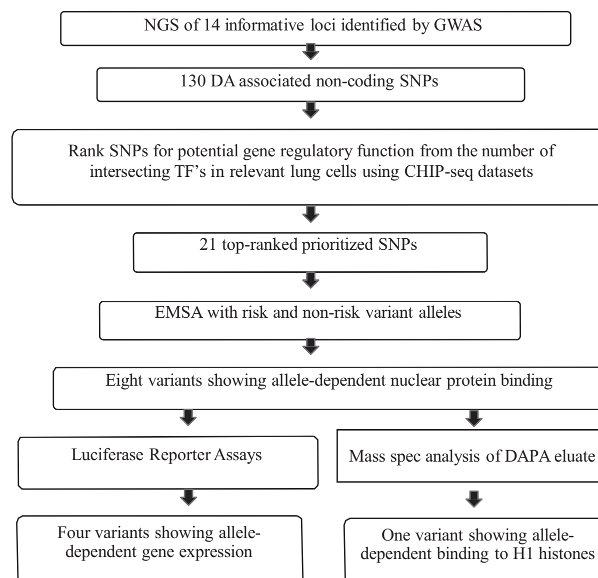

FIG 1. Study design used to identify DA-associated regulatory variants.

METHODS

Study participants

Ninety-one workers exposed to either hexamethylene diisocyanate, methylene diphenyl diisocyanate, or toluene diisocyanate with DA confirmed by positive SIC results were studied. As a comparator group, we used 238 subjects with available DNA sequence data from the 1000 Genomes control data set.[25] All subjects were of European descent. Hypothetically, healthy control subjects could develop OA if exposed to diisocyanates but likely no more than the known incidence of this disorder (2% to 5%) versus 100% of subjects with the disease.

Secondly, the absence of OA could not be ensured, even among a small number of "asymptomatic" workers without challenge testing, a procedure not considered ethical unless clinically indicated.[26] For these reasons, asymptomatic healthy control subjects have been used for this and another published GWAS of DA.[19]

Of the 91 cases, 87 underwent controlled SIC testing to a diisocyanate to prove OA conducted at 4 participating sites (2 sites in Spain and 2 sites in Quebec, Canada) in specialized laboratory facilities according to published protocols.[26-28] SIC testing was not available in 4 workers at one center (Toronto, Canada), and serial monitoring of peak expiratory flow rates was used as an alternative procedure to confirm DA.[29] These patients had the diagnosis rigorously confirmed by using serial measurements of peak flow rates recorded by workers every 4 to 6 hours and at least 4 times a day during at least 2 weeks while working and 1 to 2 weeks off work. In addition, methacholine challenge tests were performed at the end of a working week and at the end of at one week off work, showing a significant (≥3-fold) improvement in methacholine PC_{20} values away from work.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained for all subjects. The study protocol was reviewed and renewed annually by the ethics committees of participating institutions.

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