The *IL6R* variation Asp^{358}Ala is a potential modifier of lung function in subjects with asthma

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Background: The *IL6R* single nucleotide polymorphism (SNP) rs4129267 has recently been identified as an asthma susceptibility locus in subjects of European ancestry but has not been characterized with respect to asthma severity. The SNP rs4129267 is in linkage disequilibrium ($r^2 = 1$) with the *IL6R* coding SNP rs2228145 (Asp^{358}Ala). This *IL6R* coding change increases IL-6 receptor (IL-6R) shedding and promotes IL-6 transsignaling.

Objectives: We sought to evaluate the *IL6R* SNP rs2228145 with respect to asthma severity phenotypes.

Methods: The *IL6R* SNP rs2228145 was evaluated in subjects of European ancestry with asthma from the Severe Asthma Research Program (SARP). Lung function associations were replicated in the Collaborative Study on the Genetics of Asthma (CSGA) cohort. Serum soluble IL-6R levels were measured in subjects from SARP. Immunohistochemistry was used to qualitatively evaluate IL-6R protein expression in bronchoalveolar lavage cells and endobronchial biopsies.

Results: The minor C allele of *IL6R* SNP rs2228145 was associated with a lower percent predicted FEV_1 in the SARP cohort ($P = .005$), the CSGA cohort ($P = .008$), and in a combined cohort analysis ($P = .003$). Additional associations with percent predicted forced vital capacity (FVC), FEV_1/FVC ratio, and PC_{20} were observed. The rs2228145 C allele (Ala^{358}) was more frequent in severe asthma phenotypic clusters. Elevated serum soluble IL-6R levels were associated with lower percent predicted FEV_1 ($P = .02$) and lower percent predicted FVC ($P = .008$) (n = 146). IL-6R protein expression was observed in bronchoalveolar lavage macrophages, airway epithelium, vascular endothelium, and airway smooth muscle.

Conclusions: The *IL6R* coding SNP rs2228145 (Asp^{358}Ala) is a potential modifier of lung function in subjects with asthma and might identify subjects at risk for more severe asthma. IL-6 transsignaling might have a pathogenic role in the lung.

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In a recent genome-wide association study (GWAS) of 57,800 subjects from multiple asthma cohorts, the minor T allele of the IL-6 receptor *(IL6R)* single nucleotide polymorphism (SNP) rs4129267 (1q21.3) was identified as a novel asthma susceptibility locus (odds ratio, 1.09) in subjects of European ancestry.[1] This SNP was not evaluated for associated asthma severity phenotypes, including lung function, in this GWAS, but this SNP has been associated with differences in pulmonary function in healthy subjects from the Framingham Heart Study.[2]

The SNP rs4129267 is located in intron 8 of *IL6R* and is in linkage disequilibrium (LD; $r^2 = 1$) with the *IL6R* coding SNP rs2228145 (Asp^{358}Ala). The Ala^{358} variation modifies the IL-6R peptide structure adjacent to the exterior cell surface and significantly enhances proteolytic cleavage of IL-6R from cell surfaces into the extracellular space, which is termed IL-6R "shedding."[3,4] IL-6R shedding is increased in subjects who inherit the *IL6R* rs2228145 C allele (Ala^{358}) and is easily measured in serum.[5] The soluble IL-6 receptor (sIL-6R) can be activated by IL-6 and can form a complex with the ubiquitously expressed membrane-bound glycoprotein 130, resulting in activation of the IL-6 signal transduction pathway in cells that do not express membrane-bound IL-6R. This dysfunctional activation of IL-6 signaling is termed IL-6 transsignaling.[6] During IL-6 transsignaling, the sIL-6R/glycoprotein 130 complex induces tyrosine kinase Janus kinase 2 activity, which phosphorylates and activates the transcription factor signal transducer and activator of transcription 3. Activated signal transducer and activator of transcription 3 translocates to the nucleus to affect a wide range of gene expression. IL-6 transsignaling has been implicated in a range of inflammatory diseases, including rheumatoid arthritis,[7] Crohn disease,[8] and inflammatory bowel disease,[9] and is the target of anti–IL-6R therapies.[10]

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Abbreviations used
ATS: American Thoracic Society
BAL: Bronchoalveolar lavage
CSGA: Collaborative Study on the Genetics of Asthma
FVC: Forced vital capacity
GWAS: Genome-wide association study
IL-6R: IL-6 receptor
LD: Linkage disequilibrium
SARP: Severe Asthma Research Program
sIL-6R: Soluble IL-6 receptor
SNP: Single nucleotide polymorphism

In this study the *IL6R* coding SNP rs2228145 ($Asp^{358}Ala$) was evaluated in subjects of European white ancestry in 2 asthma cohorts to determine whether the coding variation is associated with lung function, an important asthma severity phenotype. Because of the wide range of lung function in the Severe Asthma Research Program (SARP) cohort, the *IL6R* coding SNP was first evaluated in the SARP cohort.[11] Lung function associations were then verified in the Collaborative Study on the Genetics of Asthma (CSGA) cohort and in a combined analysis of the SARP and CSGA cohorts. Because this coding SNP is associated with elevated serum levels of sIL-6R, SARP subjects were evaluated to determine whether correlations exist between serum sIL-6R and lung function in subjects who inherit the *IL6R* Ala^{358} isoform. The *IL6R* coding SNP was also evaluated in SARP phenotypic asthma clusters[12] to evaluate potential associations with more severe asthma. Immunohistochemistry was used to evaluate lung biopsy specimens and bronchial alveolar lavage (BAL) cells to determine whether lung cells express IL-6R protein and thus possible sources for IL-6R shedding. Finally, BAL fluid was evaluated in a small subset of SARP subjects to determine whether increased BAL sIL-6R levels were associated with inheriting the *IL6R* Ala^{358} isoform.

METHODS

Subject recruitment

Subjects of European ancestry from 9 clinical research centers that comprise the National Heart, Lung, and Blood Institute–sponsored SARP study were characterized with spirometry and were phenotyped using comprehensive standardized approaches, as previously described.[11,12] Current smokers and subjects with more than 5 pack years of smoking were ineligible for this study. In brief, subjects were defined as having severe asthma using the American Thoracic Society (ATS) workshop criteria for refractory asthma.[13] For comparison, additional subjects with nonsevere asthma were studied who did not meet the ATS workshop criteria. After appropriate withholding of bronchodilators, spirometry was performed according to ATS guidelines.[14] Hankinson values were used to calculate percent predicted FEV_1.[15] Subjects were required to have a percent predicted FEV_1 of greater than 55% to qualify for testing for bronchial responsiveness to methacholine (PC_{20}).[11] Bronchodilator reversibility was tested using a maximal protocol of up to 8 puffs of albuterol.

This study was approved by the institutional review boards at all study sites, and all patients provided informed consent to participate in this study. The replication population consisted of subjects of European ancestry from the Collaborative Study of the Genetics of Asthma at Wake Forest School of Medicine.[16,17] These subjects were studied using a similar protocol to SARP for phenotypic characterization.[11] SARP control subjects used for serum analysis had no history of asthma and no first-degree relatives with asthma.

SNP genotyping and assessment

All *IL6R* genotypes were acquired from the Illumina Human1M-Duo DNA BeadChip (Illumina, San Diego, Calif) from our previous GWAS.[18] In addition to primary analysis of rs2228145, the 9 additional *IL6R* SNPs rs6427641, rs1386821, rs6684439, rs4845618, rs8192282, rs4845371, rs4129267, rs4240872, and rs2229238 were selected for analysis based on LD (r^2) values from the HapMap database (http://hapmap.ncbi.nlm.nih.gov/). The genotyping efficiency on the Illumina Human1M-Duo DNA BeadChip for all *IL6R* SNPs was greater than 99%, and all SNPs were in Hardy-Weinberg equilibrium.

Serology and immunohistochemistry

Investigative bronchoscopy was performed on a subset of SARP subjects with all levels of asthma severity.[11] All subjects undergoing bronchoscopy were self-selected for this portion of the SARP study, and biopsy specimens analyzed were randomly selected without *a priori* selection based on asthma severity. Endobronchial biopsy specimens (n = 14) from formalin-fixed, paraffin-embedded sections (5 μm) were deparaffinized (Histoclear II; National Diagnostics, Atlanta, Ga) and rehydrated through decreasing ethanol solutions. The airway tissue was treated for antigen retrieval (Dako, Glostrup, Denmark) and blocked before incubation steps with primary IL-6Ra mAb (specific to Arg387-Arg468; R&D Systems, Minneapolis, Minn), secondary biotin-labeled anti-mouse immunoglobulin (GE Healthcare, Fairfield, Conn), streptavidin–alkaline phosphatase (Roche, Mannheim, Germany), and development with Vector Red substrate (Vector Laboratories, Burlingame, Calif). Sections were counterstained with Mayer hematoxylin.

BAL cells (n = 22) were air-dried on cytospin slides, fixed with 10% formalin for 15 minutes, and washed once with PBS before storage at −20°C. BAL cells were randomly selected from subjects who had undergone bronchoscopy, and the subset of cells was analyzed without *a priori* selection based on asthma severity. Cells were blocked with 100% FBS in PBS with 0.1% saponin, avidin, and biotin separately (Vector Blocking Kit) before incubation with primary IL-6Ra mAb (R&D Systems), secondary biotin-labeled anti-mouse immunoglobulin (GE Healthcare), streptavidin-alkaline phosphatase (Roche), and development with BCIP/NBT substrate (Dako) containing a 1:100 dilution of levamisole (Sigma, St Louis, Mo).

Serum and BAL sIL-6R levels were measured with the sIL-6R DuoSet (R&D Systems) ELISA kit and reported in ng/mL. All serum samples were diluted 1:200.

Statistical analysis

Analysis was performed by using a linear additive model adjusting for the age and sex for each population and in a combined dataset for the asthma quantitative traits percent predicted FEV_1 ($ppFEV_1$), percent predicted forced vital capacity (ppFVC), FEV_1/FVC ratio, and PC_{20} using ANOVA (2-tailed *P* values), as implemented in SAS/Genetics software (SAS Institute, Inc, Cary, NC). Serum sIL-6R measurements were analyzed using a general linear model and were log transformed to normalize sample distribution.

RESULTS

Study population characteristics

The demographic characteristics of SARP subjects with asthma in this study are described in Table I and recently have recently been extensively reported.[11] DNA from subjects of European white ancestry with asthma (n = 510, 44% with severe asthma) and without asthma (n = 45) were genotyped. Subjects with severe asthma met the ATS criteria for severe asthma and were all treated with high-dose inhaled or oral corticosteroids at recruitment, whereas 60% of subjects with nonsevere asthma