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ORIGINAL ARTICLE

Genome-Wide Association Study of Short-Acting β_2-Agonists
A Novel Genome-Wide Significant Locus on Chromosome 2 near *ASB3*

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Abstract

Rationale: β_2-Agonists are the most common form of treatment of asthma, but there is significant variability in response to these medications. A significant proportion of this responsiveness may be heritable.

Objectives: To investigate whether a genome-wide association study (GWAS) could identify novel pharmacogenetic loci in asthma.

Methods: We performed a GWAS of acute bronchodilator response (BDR) to inhaled β_2-agonists. A total of 444,088 single-nucleotide polymorphisms (SNPs) were examined in 724 individuals from the SNP Health Association Resource (SHARe) Asthma Resource Project (SHARP). The top 50 SNPs were carried forward to replication in a population of 444 individuals.

Measurements and Main Results: The combined *P* value for four SNPs reached statistical genome-wide significance after correcting for multiple comparisons. Combined *P* values for rs350729, rs1840321, rs1384918, and rs1319797 were 2.21×10^{-10}, 5.75×10^{-8}, 9.3×10^{-8}, and 3.95×10^{-8}, respectively. The significant variants all map to a novel genetic region on chromosome 2 near the *ASB3* gene, a region associated with smooth muscle proliferation. As compared with the wild type, the presence of the minor alleles reduced the degree of BDR by 20% in the original population and by a similar percentage in the confirmatory population.

Conclusions: These GWAS findings for BDR in subjects with asthma suggest that a gene associated with smooth muscle proliferation may influence a proportion of the smooth muscle relaxation that occurs in asthma.

Keywords: single-nucleotide polymorphism; genotype; bronchodilator

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*These authors contributed equally to this work.

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At a Glance Commentary

Scientific Knowledge on the Subject: Short-acting β-agonists are the most commonly used medications for asthma in the world. Familial studies have confirmed that a significant proportion of the response to these medications is heritable, yet no common genetic variant has been reported that meets genome-wide significance for association with β-agonist response.

What This Study Adds to the Field: In this study, we describe the genome-wide association and replication of four variants on chromosome 2 with the bronchodilator response to short-acting β-agonists in multiple National Heart, Lung, and Blood Institute clinical asthma trials.

Asthma is a complex respiratory disease affecting more than 300 million people worldwide and its prevalence continues to increase. It is the most common chronic disease in children (1, 2) and the most common cause of admissions to pediatric hospitals within the United States, accounting for half a million hospitalizations a year (3). A key clinical characteristic of asthma is reversible airway obstruction. Bronchodilator response (BDR) measures the change in airway constriction before and after the administration of a short-acting β_2-agonist, which acts to reduce bronchoconstriction by stimulating the β_2-adrenergic receptors on airway smooth muscle. β_2-Agonists are the most efficacious treatments for both chronic and acute asthma (1); however, there is great interindividual variability in the response to inhaled β_2-agonists. Genetics is believed to be a key component that results in varied BDR (2). Previous work by our laboratory determined heritability of BDR to vary from 10–40% (4, 5). Therefore, the identification of genetic variants that influence BDR will likely prove useful in optimizing individual asthma treatment.

BDR to β_2-agonists has been investigated as a phenotype in both candidate gene and genome-wide association studies (GWAS). Initial studies focused on the role of the β2-adrenergic receptor (*B2AR*) gene, with mixed results. A study from the Asthma Clinical Research Network (ACRN) reported adverse effects of regular albuterol treatment among subjects with asthma who were homozygous for the +49 A allele (Arg16) of the *ADRB2* gene (6–8). Associations for BDR and *ADRB2* are less conclusive. For instance, in a study of Latino Americans, the Arg16 allele was associated with BDR in Puerto Rican subjects with asthma, but not in Mexican Americans (9). Additional studies of this polymorphism in persistent asthma have failed to yield an association with BDR (10, 11), suggesting that study of the *ADRB2* locus by itself is insufficient to explain the variability of BDR in asthma.

Other candidate genes, including *CRHR2* and *ARG1*, have demonstrated replicable results (12) with BDR. For the latter, the study genotyped 844 single-nucleotide polymorphisms (SNPs) across 111 asthma candidate genes in 209 parent-offspring trios and found that four SNPs located 5′ of the arginase 1 gene (*ARG1*) were associated with BDR in three independent asthma trial populations (13). A subsequent study identified a functional haplotype at this locus that regulates gene expression and accounted for the association with BDR in these asthma trial populations (14). More recently, a GWAS analysis of BDR followed by small interfering RNA knockdown and Western blot analyses identified *SPATS2L* as an important regulator of β_2-adrenergic receptor down-regulation. Herein, we used data from the SNP Health Association Resource (SHARe) Asthma Resource Project (SHARP), an NHLBI initiative that genotyped GWAS data from three large asthma clinical trials. In this manuscript we report, for the first time, a locus with genome-wide significance, associated with variation in response to β-adrenergic bronchodilators.

Methods

Detailed methods can be found in the online supplement.

Study Populations

SHARP conducted genome-wide genotyping in adults and children who have participated in the NHLBI clinical research trials on asthma. The SHARP population included subjects who participated in three National Institutes of Health–sponsored studies: (*1*) 315 children participating in the Childhood Asthma Management Program (CAMP), (*2*) 178 children participating in the Childhood Asthma Research and Education (CARE) Network, and (*3*) 231 adults participating in the ACRN. The ascertainment criteria and study designs for each of the studies conducted by these networks were comparable, although not identical. We limited our population to white individuals to avoid potential confounding due to population stratification. Further information on the SHARP project can be found in the online supplement and at the database of Genotypes and Phenotypes website (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000166.v2.p1). In each of these studies we identified subjects who had FEV_1 assessed before and after administration of at least two puffs of inhaled albuterol.

Genotyping and Quality Control Measures

Detailed quality control, including specific information for each individual trial, is described in the online supplement. GWAS genotyping was performed using the Affymetrix (Santa Clara, CA) genome-wide human SNP Array 6.0 including 906,600 SNPs. Marker QC was performed on all autosomal markers extracted from the database of Genotypes and Phenotypes for each of the three cohorts separately. Details of the principal component analysis with EIGENSTRAT software (15) was performed among participating white subjects to evaluate potential population substructures; 62 subjects were removed from the analysis because of potential population stratification (9 from CAMP, 21 from CARE, and 32 from ACRN). Additional details are included in the online supplement.

Outcome Phenotype

The primary outcome phenotype was the change in FEV_1 after two or more puffs of albuterol expressed in one of two ways: FEV_1 post-bronchodilator minus FEV_1 prebronchodilator where the FEV_1 was expressed as a percent of the predicted FEV_1 adjusted for puffs of bronchodilator administered (two puffs for ACRN, >2 puffs for CAMP and CARE); and (FEV_1

post-bronchodilator minus FEV_1 prebronchodilator)/prebronchodilator FEV_1. Although the latter is the definition conventionally applied to define BDR in the clinical setting, the former may be a more stable measure of BDR overall (16). A total of 724 white subjects were available with BDR. The use of two distinct, yet related phenotypes was one of the strategies that was incorporated in this study to reduce the variability in the phenotypic response to drug.

Replication Strategy for SNPs

Using *P* values, we identified the top 100 SNPs that were associated with the two BDR definitions (*see* the OUTCOME PHENOTYPE section). We identified the SNPs that were common to these two lists and restricted the analysis to the 50 SNPs on the common list that were most strongly associated with β-agonist response. The combined *P* value for each of these 50 SNPs was less than or equal to 2×10^{-4}. We genotyped these SNPs in the replication population sample described next. Replication population genotyping was performed using the Sequenom platform (Sequenom, San Diego, CA). Of the 50 SNPs submitted for genotyping, 42 (82%) were successfully genotyped and available for analysis.

Replication Population

All participants (n = 439) were diagnosed with moderate to severe asthma according to the American Thoracic Society criteria (17), had no significant comorbid medical conditions, and were not taking any other asthma medications during the trial. No oral or inhaled corticosteroids were administered during the 6 weeks leading up to the trial. Entry criteria included an FEV_1 of 40–85% of predicted normal value after at least 8 hours without use of an inhaled short-acting β-agonist, and a minimum of 15% of baseline improvement in the FEV_1 in response to the β-agonist.

Statistical Analysis

Genome-wide association analyses were performed using PLINK (18). For the primary analysis, the two pediatric populations (CAMP and CARE) were pooled. The two definitions for BDR were the phenotypes used in the analysis. Analyses were adjusted for age, height, sex, number of puffs, and the six principal components for population stratification. Trial-specific β estimates were generated for the two pediatric cohorts together and ACRN to ascertain the consistency of the findings. The data were then pooled together, with the same covariates as well as an additional variable for study. This yielded the combined SHARP *P* value. β estimates and standard errors from the CAMP/CARE and ACRN analysis were then used to create a combined β estimate, using a random-effects approach (19). This is a widely used and well-accepted metaanalytic method for combining information across studies.

Analysis in the replication population consisted of generalized linear models evaluating the association between each of the selected SNPs and change in FEV_1 while adjusting for age, sex, and height using SAS (version 9.1; SAS Institute, Cary, NC). All genotypes were modeled additively and the genotypic means were calculated using least square means to adjust for possible confounders. A replication of the initial finding was defined by having a nominal *P* value less than 0.05 in the replication cohort with an effect estimate in the correct direction. Stouffers Z-transform test (20) was used to calculate a *P* value for overall association combining the *P* values for the CAMP/CARE/ACRN and the replication population together.

Results

Subjects

A total of 724 white subjects were included in the phenotypic and genotypic analysis from ACRN, CAMP, and CARE (Table 1). Males represented most subjects in the pediatric (CARE and CAMP) populations as opposed to the ACRN population. The population characteristics for the replication population are also shown in Table 1. This population, which was chosen for its BDR response and whose entry criteria included an upper bound on FEV_1, had a lower baseline FEV_1 than the SHARP populations and a much larger BDR. The age of the replication population individuals was comparable with subjects in ACRN.

GWAS in the SHARP Population

After excluding SNPs with low minor allele frequencies (<5%; 154,218 SNPs) and considering SNPs that were available in all three samples (624,229 SNPs), a total of 444,088 SNPs were available for analysis. The association between the 444,088 SNPs and the change in FEV_1 (as expressed as change in FEV_1 as a percent of baseline FEV_1 and as percent of predicted FEV_1) in response to albuterol is displayed on Manhattan plots (Figures 1A and 1B). There was 85% overlap between the two methods of expressing BDR. As described in the METHODS section, the top 50 SNPs (based on lowest *P* values) were selected for replication. In total, 42 of the 50 SNPs passed genotype quality control in the replication population. From the 42 SNPs that were genotyped, five SNPs were associated ($P < 0.05$) with the effect estimate in the same direction as the combined SHARP association results.

Table 2 lists the *P* value and effect estimate for CAMP/CARE, ACRN, the combined SHARP population, and the replication population. In addition, the overall combined *P* value for SHARP and the replication population is presented. Although the SNPs were screened using two different BDR phenotypes, the replication analysis, including these five SNPs, included only SNPs both within the top 100 SNPs and associated with both of the BDR phenotypes. Of the five SNPs that replicated in the replication population, the combined *P* value for four of the five SNPs reached statistical significance after correcting for multiple comparisons (the combined *P* values for rs350729, rs1840321, rs1384918, and rs1319797 were 2.21×10^{-10}, 5.75×10^{-8}, 9.3×10^{-8}, and 3.95×10^{-8}, respectively).

To elucidate potential differences related to baseline FEV_1, we reanalyzed our top four SNPs within the SHARP cohort. Each of the SNPs remained significant following adjustment for baseline FEV_1 at a *P* value nearly identical to that of the initial GWAS analysis (*see* Table E1 in the online supplement). All five of the replicated SNPs are in a singular region of chromosome 2p16.2. There was modest linkage disequilibrium between the SNPs (Figure 2). The nearest coding gene is the gene coding for ankyrin repeat (*ASB3*) and *SOCS* (suppressor of cytokine signaling) box-containing protein 3. For each of the significant SNPs (rs350729, rs1840321, rs1384918, and rs1319797) there was approximately a 20% decrease in mean BDR response in those homozygous for the minor allele compared with those homozygous for the major allele (Figure 3).

Table 1. SHARP and Replication Population Characteristics

	CAMP	CARE	ACRN	Replication
Sample size	315	178	231	439
Age (SD)	8.7 (2.1)	10.5 (2.9)	32.0 (10.5)	32.3 (13.6)
% Male	193 (61%)	111 (62%)	96 (42%)	223 (50%)
Baseline FEV_1 (% predicted)	93.1 (14.5)	99.3 (12.6)	85.8 (13.5)	61.4 (6.9)
Change in FEV_1 as a % of baseline	11.0 (10.6)	10.4 (8.4)	11.0 (10.2)	40.2 (21.0)

Definition of abbreviations: ACRN = Asthma Clinical Research Network; CAMP = Childhood Asthma Management Program; CARE = Childhood Asthma Research and Education; SHARP = SNP Health Association Resource (SHARe) Asthma Resource Project.
For the primary genome-wide association study analysis, CAMP and CARE were pooled. Analyses were run in the pooled samples and in ACRN. Combined β estimates were generated using a random-effects model.

To further assess the specific relationship of our top SNP, rs350729, with *ASB3*, we performed two informatics database analyses. First, we evaluated ENCODE Chip-Seq data in close proximity to the variant, using the UCSC Genome Browser. This demonstrated two transcription factors, *JUND* and *CEBPB*, in close proximity to the SNP. We also evaluated the role of rs350729 as a potential expression quantitative trait locus, using the SCAN database (http://www.scandb.org). Rs350729 was associated with the expression of 52 genes at a *P* value less than 1×10^{-4}. The genes regulated by rs350729 in turn demonstrate prominent interactions with both *ASB3* and *ADRB2* (*see* Figure E1).

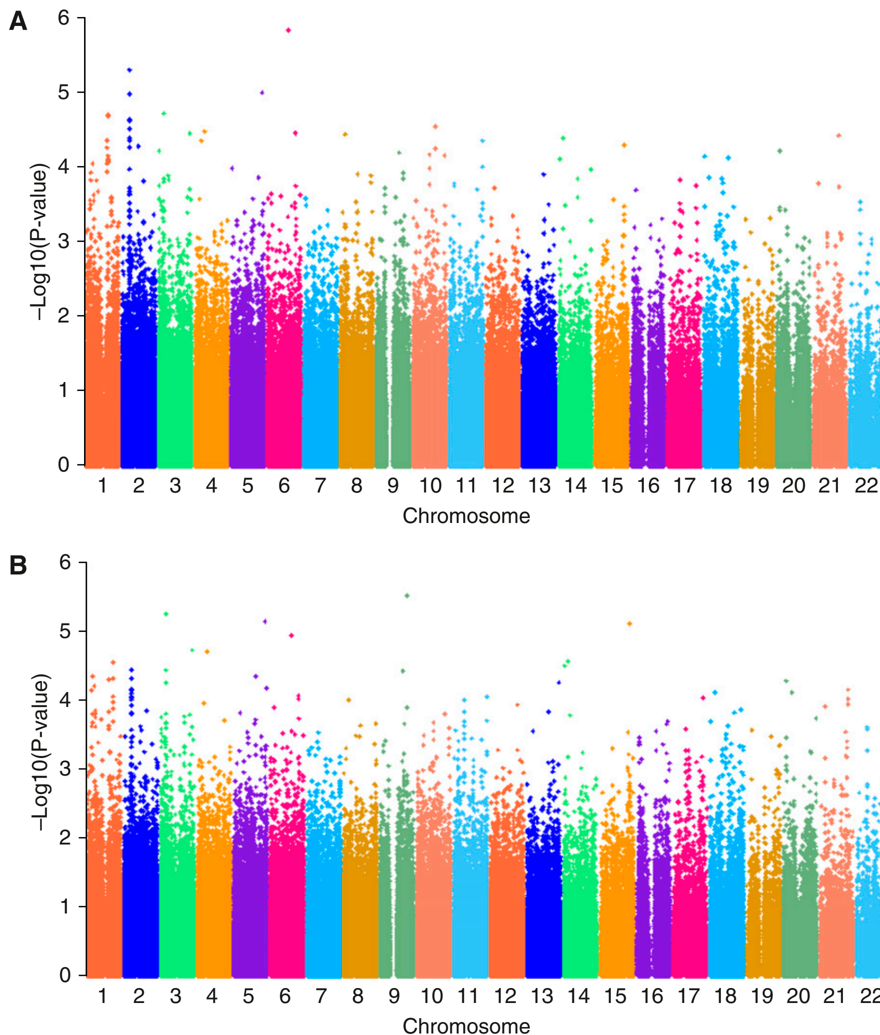

Figure 1. Plot of *P* values by chromosome for change in FEV_1 in response to bronchodilator. (*A*) Change in FEV_1 as a percent of predicted. (*B*) Change in FEV_1 as a percent of baseline FEV_1.

Discussion

The improvement in FEV_1 after administration of a β-agonist, the BDR, is used as a diagnostic criterion for asthma (21, 22). In subjects with asthma, BDR has been shown to predict responsiveness to inhaled corticosteroid medications (23, 24). Persistently high BDR has also been associated with increased risk for future exacerbations (25). Therefore, knowledge of genetic factors associated with BDR has potential diagnostic and prognostic implications.

We performed a GWAS analysis on bronchodilator phenotypes in the SHARP population. We then identified the top genetic associations that were common to the GWAS analysis and found that three of these SNPs associated with BDR in a replication population at a level of genome-wide significance. All of these SNPs were located in the same region on chromosome 2, suggesting that we have identified a novel genomic region associated with the acute BDR.

Of these significant associations, all three had an approximate 20% increase in the BDR when comparing homozygosity of the two major alleles with the two minor alleles. Therefore, on average, subjects homozygous for the minor allele would have a lower BDR versus those homozygous for the wild-type allele. The absolute difference was most apparent in the replication population because the baseline BDR was greater in that population. We report (Table 2) two additional SNPs within the same region that do not meet criteria for genome-wide significance, but have similar effect estimates. One of these, rs785291, is associated in the opposite direction of the other four SNPs and is not in linkage disequilibrium (R^2 of 0.40) with the other variants. This supports both a true independent effect and enhances the likelihood that this region in general serves as a regulatory locus influencing BDR.

Table 2. Replicated SNPs among the Top 42 SHARP *P* Values for FEV_1 Change

SNP	Chr	Base Pair	Allele	CAMP/CARE β Estimate	CAMP/CARE *P* Value	ACRN β Estimate	ACRN *P* Value	SHARP Combined *P* Value	SHARP Combined β Estimate	Replication β Estimate	Replication *P* Value (One-sided)	Overall *P* Value
rs350729	2	52837277	4	−2.22	3.3×10^{-4}	−3.65	4.16×10^{-4}	3.04×10^{-7}	−2.26	−5.63	7.45×10^{-5}	2.21×10^{-10}*
rs1840321	2	52814911	2	−1.91	1.41×10^{-3}	−2.28	1.96×10^{-2}	4.71×10^{-5}	−1.59	−5.17	8.5×10^{-5}	5.75×10^{-8}*
rs1384918	2	52827838	3	−1.86	1.83×10^{-3}	−2.29	1.94×10^{-2}	6.24×10^{-5}	−1.57	−5.08	1.07×10^{-4}	9.3×10^{-8}*
rs1319797	2	52822533	3	−2.00	9.62×10^{-4}	−2.40	1.44×10^{-2}	2.28×10^{-5}	−1.66	−5.00	1.52×10^{-4}	3.95×10^{-8}*
rs785291	2	52871781	2	1.99	1.23×10^{-4}	2.04	3.39×10^{-2}	5.04×10^{-5}	1.64	3.67	4.21×10^{-3}	1.34×10^{-6}

Definition of abbreviations: ACRN = Asthma Clinical Research Network; CAMP = Childhood Asthma Management Program; CARE = Childhood Asthma Research and Education; Chr = chromosome; SHARP = SNP Health Association Resource (SHARe) Asthma Resource Project; SNP = single-nucleotide polymorphism.
*Significant at $\alpha = 0.05$ after correction for multiple comparisons.

The nearest gene to the significant associations observed in this analysis is the gene coding for ankyrin repeat (*ASB3*) and *SOCS* (suppressor of cytokine signaling) box-containing protein 3. Although this gene is almost a megabase from the SNPs we have identified, the proteins encoded by the *ASB3/SOCS* genes have several properties that suggest a possible physiologic role that might explain their association with BDR. First, ASB-related genes have been shown to be primarily expressed in skeletal muscle and their expression seems to be affected by β-agonists (26). Furthermore, these genes have been shown to participate in the regulation of protein turnover and muscle cell development by stimulating protein synthesis and regulating differentiation of muscle cells (27). Furthermore, mouse studies have suggested that this gene family may have a role in myoblast differentiation (28). Lastly, Ankyrin repeat-containing proteins control the induction of hypertrophic responses in muscle in response to loads (29). These muscle regulatory functions could potentially explain the effect on the acute BDR, which is highly dependent on airway muscle function.

In addition to its possible role in airway muscle, interrogation of *ASB3* in an asthma case-control analysis (30) within the publicly available GEO database (http://www.ncbi.nlm.nih.gov/geo/) (GDS1269) suggests a possible relationship with asthma affection status in alveolar macrophages ($P = 0.05$). From a pathway perspective, *ASB* family members regulate phosphorylation of MAP-kinase in the Erk1/2 signal transduction pathway and of Akt in the PI3K/Akt signal transduction pathway (28). In turn, Erk1/2 and PI3K/Akt form two key mediators for airway smooth muscle proliferation (31). *ADRB2* negatively interacts with Erk1/2 (32), which may explain the relationship between *ASB3* and the *ADRB2* mediated BDR. Additionally, a BioGrid (http://thebiogrid.org/) interaction database analysis reveals multiple genes that interact with both *ASB3* and *ADRB2*. When visualized via GeneMania (33) (www.genemania.org), a close relationship between these two genes is apparent (*see* Figure E2).

In addition to a potential direct regulatory relationship with *ASB3*, the SNPs identified may influence BDR in other possible ways. We noted that the top SNP, rs350729, is in close proximity to two transcription factors, *JUND* and *CEBPB*. *JUND* has not been implicated in asthma, but may play a prominent role in cardiac hypertrophy and drug response (34), supporting a role in muscle function. Although *CEBPA* is commonly implicated in airway smooth muscle proliferation, *CEBPB* can regulate *CEBPA* (35) and is up-regulated by the β-agonist formoterol (36). Our top SNP also seems to be a transcriptional hotspot in that it is significantly associated with expression of 52 genes. In turn these regulated genes may interact with the *ASB3* and *ADRB2* pathways (*see* Figure E1), supporting an additional mechanism linking both this chromosomal 2 region and the *ASB* gene with *ADRB2*-mediated bronchodilation.

There were several novel approaches undertaken in this GWAS analysis. First, we

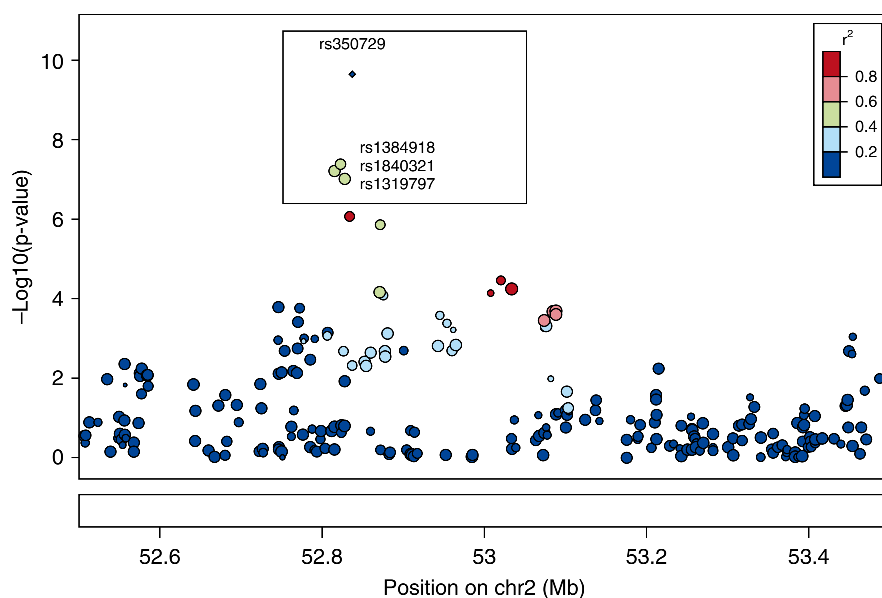

Figure 2. Top single-nucleotide polymorphisms in bronchodilator-response association study. The Locus Zoom (http://csg.sph.umich.edu/locuszoom/) plot shows the linkage negative log *P* values and linkage disequilibrium patterns of the top four single-nucleotide polymorphisms in the combined analysis of the SHARP population and the replication "asthma population." chr2 = chromosome 2.

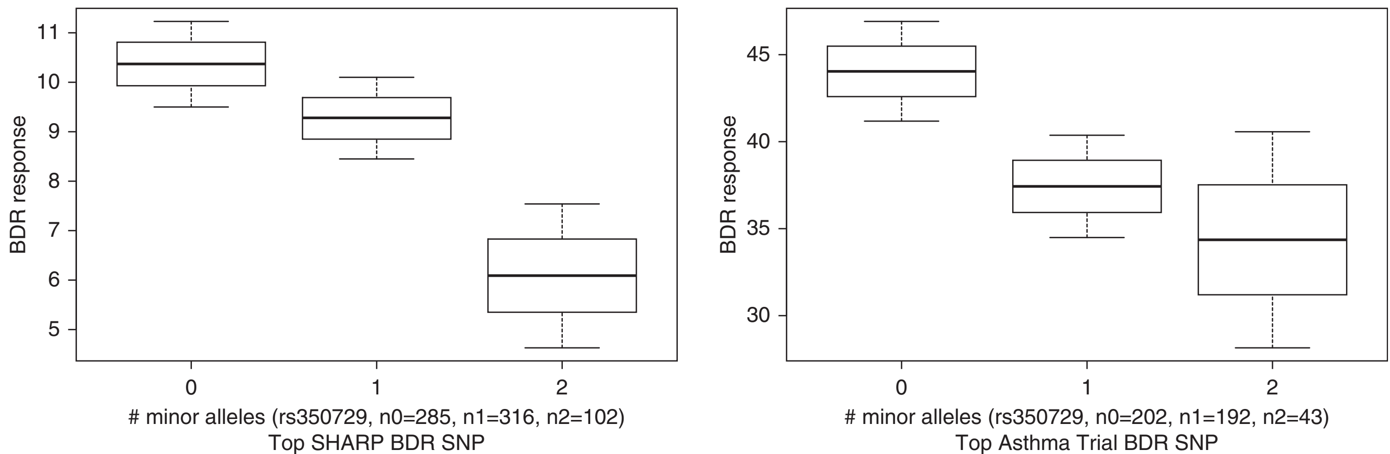

Figure 3. Least-squares means plots of FEV_1 in response to bronchodilator by the number of copies of the minor allele for the SHARP and Asthma Trial populations. BDR = bronchodilator response; SHARP = SNP Health Association Resource (SHARe) Asthma Resource Project; SNP = single-nucleotide polymorphisms.

specifically chose to look at two distinct, yet closely related BDR phenotypes and then identify the consistent association findings between the two phenotypes. This approach was deliberate, because it is known that there is strong individual variation in BDR response. Therefore, we deliberately used two BDR phenotypes for each individual to reduce this individual variation and increase the validity of our findings. This approach worked well, because we were able to identify genetic associations that reached genome-wide significance for the first time.

The current analysis focuses on response to short-acting β-agonists. However, the loci identified would likely also influence the response to long-acting β-agonists. Those studies, however, would also likely involve additional loci, because long-acting β-agonists are concomitantly administered with inhaled corticosteroid preparations in asthma. Identification of loci related to response to long-acting β-agonists has begun (37, 38), although the studies to date have focused exclusively on the *ADRB2* gene and no GWAS study has been published.

We and others have previously reported associations of multiple candidate genes with the acute BDR (9, 11–14, 39, 40). Until recently, no regions of the genome had been associated with BDR in a GWAS. Using GWAS, we have reported that the SNP rs295137 near the *SPATS2L* gene was associated with acute BDR in multiple asthma cohorts, with an overall combined P value of 9.7×10^{-07}. Although this value did not quite reach genome-wide significance, investigation of the *SPATS2L* messenger RNA knockdown showed increased β2-adrenergic receptor levels, suggesting that *SPATS2L* may be an important regulator of the β2-adrenergic response. Although the *SPATS2L* variant was not genotyped on the platform used in this analysis, other SNPs in this region were associated with BDR in this analysis (e.g., rs4233996; $P = 6.4 \times 10^{-4}$). Using a gene-based GWAS approach, Padhukasahasram and colleagues (41) have identified a gene, *SPATA13-AS1*, associated with BDR in multiple cohorts, although no individual SNP demonstrated consistent effects. Another recent genome-wide study identified associations with BDR in a large combined cohort of Latinos and Hispanics. In this case, all the SNPs identified had a minor allele frequency of less than 3%, suggesting that future studies directed at rare variants may add additional insights into the biology underlying asthma treatment response (42).

Nevertheless, given the rarity of the individual SNPs and potential for spurious findings from the resultant genetic association results (43), we have limited our analysis to variants with allele frequencies of greater than 5%. Notably, none of the GWAS studies on BDR to date, including the present study, has reported *ADRB2* as a top locus, suggesting that the identified associated genes likely play a regulatory role and interact with the primary β2-agonist receptor. This is consistent with potential interactions both at the SNP and *ASB3* gene level as shown in Figures E1 and E2. Our current study builds on what is known about BDR on a genome-wide level and is the first study to identify genome-wide significant associations for SNP variants associated with BDR. The specific region on chromosome 2 represents a novel location for BDR and associations with a gene with a strong biologic candidate involved in the regulation of smooth muscle responses to β-agonists *in vitro*. Moreover, these findings continue to add to the evidence that therapeutic response to asthma medications is heritable and that prediction of therapeutic response in asthma remains a tenable goal. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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