A genome-wide association study of bronchodilator response in asthmatics

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Reversibility of airway obstruction in response to β2-agonists is highly variable among asthmatics, which is partially attributed to genetic factors. In a genome-wide association study of acute bronchodilator response (BDR) to inhaled albuterol, 534,290 single-nucleotide polymorphisms (SNPs) were tested in 403 white trios from the Childhood Asthma Management Program using five statistical models to determine the most robust genetic associations. The primary replication phase included 1397 polymorphisms in three asthma trials (pooled n = 764). The second replication phase tested 13 SNPs in three additional asthma populations (n = 241, n = 215 and n = 592). An intergenic SNP on chromosome 10, rs11252394, proximal to several excellent biological candidates, significantly replicated (P = 1.98 × 10⁻⁷) in the primary replication trials. An intronic SNP (rs6988229) in the collagen (COL22A1) locus also provided strong replication signals (P = 8.51 × 10⁻⁷). This study applied a robust approach for testing the genetic basis of BDR and identified novel loci associated with this drug response in asthmatics.

The Pharmacogenomics Journal (2014) 14, 41–47; doi:10.1038/tpj.2013.5; published online 19 March 2013

Keywords: albuterol; asthma; bronchodilator response; genome-wide association study; pharmacogenetics

INTRODUCTION

Asthma is a complex respiratory disease characterized by hyper-responsiveness of the bronchial muscles, chronic inflammation and reversible narrowing of the airways. It affects ~300 million individuals worldwide and its prevalence is expected to increase to 400 million by 2025.1 Asthma is the most common chronic illness in children,2,3 accounting for half a million hospitalizations a year in the United States. In 2007, asthma-related health care costs in the United States were estimated to be $56 billion, with the majority attributed to medications and hospitalizations.4 Taken together, asthma has a significant public health impact and steps towards its prevention or better management will decrease the overall disease burden.

β2-agonists are the most commonly used drugs for treating asthma.2 The therapeutic effects result from binding to the transmembrane β2-adrenergic receptor (β2-AR) located on airway smooth muscle cells to relieve bronchoconstriction. These are available as short-acting β2-agonists (for example, albuterol) for rescuing acute asthma symptoms or as long-acting β2-agonists (for example, salmeterol and formoterol) for controlling chronic asthma that is usually administered in combination with an inhaled corticosteroid. The reversibility of airway obstruction in response to these medications, known as bronchodilator response (BDR), may be measured as a change in lung function (forced expiratory volume in one second (FEV1)) or as fall in peak expiratory flow rate, indicating a downregulation of β2-agonist responsivity (tachyphylaxis) with prolonged drug use. Inter-individual variability in response to these drugs have been previously described and research suggests that genetic variants are major contributing factors.5 The identification of genetic loci associated with BDR to β2-agonists will help in the facilitation of personalized asthma treatment regimens.

Pharmacogenetic investigations of BDR have identified a number of genetic associations for this variable drug response. The majority had been candidate gene studies, which reported genetic associations to single-nucleotide polymorphisms (SNPs) and/or haplotypes in the arginine 1 (ARQ1) locus,6 the β2-adrenergic receptor (ADRB2) gene,7–9 the corticotropin-releasing hormone receptor (CRHR)-2 locus10 and the adenylyl cyclase type 9 (AC9) gene.11 A recent genome-wide association study (GWAS) of BDR by our group identified a functional variant in the serine-rich 2-like (SPATS2L) gene, albeit the mechanism by which it regulates BDR remains unknown.12 In this manuscript, we expand on the previous literature by using a novel approach to identify genetic associations with BDR (defined by a change in lung function), whereby we apply five statistical models in a GWAS of this drug-response phenotype to decrease the likelihood of false-positive associations. Novel aspects of the current GWAS include use of genetic data from the parents of asthmatics in a family-based test, which is more robust against population stratification.
as well as analysis of 11 BDR measures for each subject taken over a 4-year period in addition to BDR at randomization (taken upon entry into the clinical trial). Moreover, we considered both additive and recessive transmissions of the associated alleles. We then pooled the results from these multiple genome-wide analytical models to identify common genetic association signals to carry forward for replication analysis in additional asthma populations. This manuscript describes the findings of our innovative GWAS of BDR in asthmatic subjects.

**SUBJECTS AND METHODS**

**Asthma trial populations**

The asthma trial populations are summarized in Table 1 and details are available in the Supplementary Material. All patients or their legal guardians consented to each study protocol and ancillary genetic testing. All studies were approved by the respective Institutional Review Boards and/or Ethics Committees of the participating institutions.

**Initial GWAS population.** A total of 403 non-Hispanic white asthmatic children and their parents from the Childhood Asthma Management Program (CAMP)\(^\text{13,14}\) were successfully genotyped on the Illumina HumanHap550v3 BeadChip (San Diego, CA, USA).\(^\text{15}\) BDR at randomization was conducted for each proband upon entry into the trial, following two inhalations of albuterol. A total of 11 longitudinal BDR values were measured in a subset of 171 asthmatics randomized to inhaled albuterol therapy as needed over 4 years of this clinical trial. Genome-wide association analysis included 534,290 autosomal SNPs that had passed quality control metrics (see Supplementary Material).

**Primary replication populations.** A total of 1356 SNPs were selected for genotyping, of which 1397 were successful, in three non-Hispanic white adult asthma trials (pooled \(n = 764\)) using the Illumina GoldenGate Custom Array (Illumina Inc., San Diego, CA). SNP selection criteria are detailed below (Statistical methodology). These replication populations included (1) the Asthma Trial (AT, \(n = 444\))^\text{16,17}\), (2) the Leukotriene modifier or Corticosteroid or Corticosteroid Salmeterol (LOCCS, \(n = 165\)) trial;\(^\text{18}\) and (3) the Effectiveness of Low Dose Theophylline as Add-on Treatment in Asthma (LODO, \(n = 155\)) trial.\(^\text{19}\) BDR at randomization was conducted for each subject upon entry into these clinical trials.

**Secondary replication populations.** A total of 13 SNPs with one-sided \(P\)-values < 0.05 (based on the direction of association in CAMP) in the primary replication analysis were further tested in two additional asthma trials: (1) the Childhood Asthma Research and Education Network (CARE, \(n = 215\)) and (2) the Asthma Clinical Research Network (ACRN, \(n = 241\)).\(^\text{20}\)

As these individuals had been genotyped on the Affymetrix Genome-Wide Human SNP Array 6.0 (Santa Clara, CA, USA), imputed data were used, which were generated for the HapMap Phase 2 Release 22 SNPs\(^\text{21}\) by applying the Markov Chain Haplotyping (MaCh) software.\(^\text{22}\) Finally, 8 of these 13 SNPs were further tested in the Genetics of Asthma in Costa Rica Study (GACRS), which were successfully genotyped on the HumanOmniExpress-12v1.\(^\text{1}\) A chip,\(^\text{23}\) BDR at randomization was conducted for each subject upon entry into these clinical trials.

**Statistical methodology**

The primary outcome measure of all analyses was BDR to the inhaled \(I_L\)-agonist albuterol, which was calculated as the percent change in forced expiratory volume in one second (FEV\(_1\)); BDR = 100 \times (\text{postFEV}_1 – \text{preFEV}_1)/\text{preFEV}_1), where preFEV\(_1\) is the lung function before albuterol treatment (baseline) and postFEV\(_1\) is the lung function following albuterol treatment. The overall analysis strategy is presented in Figure 1. To compensate for the limited statistical power given the small sample size of the CAMP trial, we used five statistical models to identify the most robust genetic associations: generalized linear model of BDR in 403 probands, using recessive (1) and additive (2) models; mixed model of 11 repeated measures of BDR over 4 years in 171 individuals randomized to as-needed inhaled \(I_L\)-agonist, using recessive (3) and additive (4) models; and family-based association test (FBAT) of BDR at randomization in 403 CAMP parent–offsprings (5). All models were adjusted for age, sex and baseline preFEV\(_1\), and model 5 was additionally adjusted for height. Each SNP was given a score of 0–5 based on the total number of \(P\)-values < 0.05 from the five association tests. SNPs scoring 5 (\(n = 437\)) were carried forward for genotyping in the primary replication cohort, but those scoring 4 were then ranked according to their \(P\)-values from the FBAT analysis as this model is robust against population stratification. No SNPs scoring < 4 were included for replication. Tests using generalized linear (additive and recessive) models were performed in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/),\(^\text{24}\) and included SNPs with minor allele frequencies \(\geq 0.05\). FBAT applied a pedigree-based analysis tool previously described.\(^\text{25}\)

All replication analyses used a single measure of BDR at randomization, with adjustments for age, sex, height and baseline preFEV\(_1\). Multiple comparisons were adjusted using the Liptak weighted \(Z\) method.\(^\text{26}\) Additional details are available in the Supplementary Material.

**Expression quantitative trait analysis**

Microarray data from immortalized lymphoblastoid cell lines of 117 asthmatics (non-Hispanic white CAMP subjects), spotted on the Illumina HumanRef8v2 microarray BeadChips, were used to test the correlation of genetic variants with gene expression. These cells were cultured and treated with ethanol (sham) as a control for differential analysis with corticosteroid (dexamethasone)-treated cells for a separate pharmacogenetic investigation (unpublished data). The microarray data from the sham arm of this experiment was vst-transformed and quantile-normalized using the ‘lumi’ package in Bioconductor.\(^\text{27}\) A \(cis\)-expression quantitative trait locus was defined as a SNP that was correlated with the expression of a gene within 50 kb. A trans-exression quantitative trait locus was a SNP correlation with a transcript located >50 kb away on a separate chromosome entirely.

**RESULTS**

The baseline characteristics of all asthma populations including CAMP, the three primary replication trials and the three secondary replication tests are presented in Table 1.

**Table 1. Baseline characteristics of participants in the asthma populations used in this analysis**

|                | CAMP (albuterol arm) | AT | LOCCS | LODO | ATLOCCS/LODO | CARE | ACRN | GACRS |
|----------------|----------------------|----|-------|------|-------------|------|------|-------|
| \(n\)           | 403                  | 171| 444   | 165  | 155         | 764  | 215  | 241   | 592  |
| Age, mean (s.d.)| 8.8 (2.1)            | 8.7 (2.1) | 32.4 (13.6) | 34.4 (15.3) | 42.9 (14.7) | 34.9 (14.8) | 10.6 (2.9) | 31.7 (42.2) | 9.0 (1.8) |
| Range           | 5.2–13.2             | 5.2–13.2 | 12.0–80 | 7–71 | 15–76       | 7–80 | 6–17.8 | 12.4–63.7 | 6.0–14.2 |
| Gender, male, n(%)| 254 (63)             | 109 (60) | 222 (50.0) | 58 (35.2) | 39 (25.2) | 319 (41.8) | 132 (61.0) | 101 (41.9) | 351 (59.3) |
| Wash out prior to BDR test, weeks | 4                     | 4 | 6 | 4 (Fucitesacone) | 2 | 2–6 | 0–4 | 0–6 | 2 |
| Albuterol puffs (90 ug per puff) | 2                    | 2 | 2 | 3 | 4 | 2 | 2 | 2 |
| pre-BD FEV\(_1\), mean (s.d.) | 93.4                 | 94.7 (13.3) | 61.5 (6.8) | 84.3 (12.3) | 78.8 (17.7) | 69.8 (14.7) | 99.3 (12.6) | 85.9 (13.5) | 99.8 (17.2) |
| BDR, mean (s.d.) | 11 (10)              | 12 (11) | 40.15 (20.9) | 6.4 (6.1) | 9.7 (11.1) | 26.7 (23.2) | 9.5 (8.4) | 11.6 (21.8) | 5.7 (9.2) |

Abbreviations: ACRN, Asthma Clinical Research Network; AT, Asthma Trial; BDR, bronchodilator response; CAMP, Childhood Asthma Management Program; CARE, Childhood Asthma Research and Education Network; GACRS, Genetics of Asthma in Costa Rica Study; LOCCS, Leukotriene modifier or Corticosteroid or Corticosteroid Salmeterol; LODO, Effectiveness of Low Dose Theophylline as Add-on Treatment in Asthma; pre-BD FEV\(_1\), pre-bronchodilator FEV\(_1\); percent predicted.

*Subjects were permitted to use rescue medications as needed during the wash-out period.*
replication populations are shown in Table 1. The distribution of BDR at randomization across all asthma trials is depicted in Figure 2. Although the initial GWAS using CAMP consisted of childhood asthmatics, the replication populations included both childhood (CARE and GACRS) and adult asthmatics (pooled AT/LOCCS/LODO and ACRN). It is also notable that the adult asthma populations had fewer males and lower pre-bronchodilator FEV\textsubscript{1} than the childhood populations (CARE and GACRS).

Genome-wide analysis in CAMP
A plot of the −log\textsubscript{10}(P-values) against the chromosomal location of each SNP from the family-based association (FBAT) analysis is shown in Figure 3. A quantile–quantile plot of the expected P-values of the FBAT analysis under the null hypothesis and the actual observed P-values illustrates that the majority of P-values were greater than expected by chance, suggesting that the test was conservative (Supplemental Figure 1). However, there are several P-values less than what was expected by chance. For example, the lowest P-value was 5.28 × 10\textsuperscript{-7} for rs8112048 located 3′ of the zinc finger protein 14 (ZNF14) gene, but this did not meet genome-wide significance. In addition, we noted that many SNPs in previously implicated genes (ARG1, ADRB2, CRHR-2 and AC9) were absent from our GWAS due to differences in genotyping platforms. Of the four markers included in our GWAS (rs1042713 in ADRB2, rs4723002 and rs226716 in CRHR2, and rs2230739 in AC9), nominal association was found for rs1042713 in ADRB2 (P<0.02), which is the most investigated locus for BDR. Finally, the genomic inflation factor estimate was 1.01, demonstrating minimal population stratification.

Replication analyses
Data for the 1397 replication SNPs from the three adult asthma trials were pooled for analysis to maximize the statistical power for detecting associations. A total of 13 SNPs replicated in the same direction as the initial GWAS population (CAMP) and were carried forward for analysis in the secondary replication phase (Table 2). The intergenic SNP, rs11252394, with a P-value of 0.0099 (β = 3.1) from the additive model in CAMP, had a one-sided P-value <0.05 in the primary replication phase, which remained significant.
significant following Bonferroni correction for multiple comparisons. However, this SNP did not replicate in the secondary replication phase. Next, nominal association signals ($P$-values $< 0.05$) were derived for an intronic SNP, rs6988229, in the collagen type XXII α 1 (COL22A1) gene in CAMP (recessive $P$-value $= 0.0004$, $\beta = 3.26$). This SNP further replicated across all asthma populations except for CARE (Liptak combined $P = 8.51 \times 10^{-6}$). Finally, five additional SNPs showed marginal association

**Table 2.** Summary of GWAS and replication analyses in all asthma clinical trials

| SNP          | Chr | MA | MAF  | Gene   | Model  | CAMP GWAS ($P$-value) | Replication 1 ($P$-value) | Replication 2 ($P$-value) | Liptak combined ($P$-value) |
|--------------|-----|----|------|--------|--------|-----------------------|---------------------------|---------------------------|-----------------------------|
| rs11252394   | 10  | A  | 0.08 | Additive | 0.0099 ($+)$ | 1.21E-06 | 0.4173 | 0.3472 | — | 1.98E-07 |
| rs6988229    | 8   | T  | 0.20 | COL22A1 | Recessive | 0.0004 ($+)$ | 0.0050 | 0.5050 | 0.9283 | 0.0139 | 8.51E-06 |
| rs9552679    | 13  | C  | 0.26 | Additive | 0.0003 ($+)$ | 0.0004 | 0.7864 | 0.4410 | — | 3.02E-05 |
| rs1663330    | 14  | G  | 0.33 | Additive | 0.0200 ($+)$ | 0.0028 | 0.0234 | 0.7544 | — | 4.54E-05 |
| rs1663332    | 14  | T  | 0.37 | Additive | 0.0028 ($+)$ | 0.0006 | 0.0374 | 0.6874 | 0.3595 | 8.07E-05 |
| rs17495520   | 5   | T  | 0.14 | Additive | 0.0338 ($+)$ | 0.0110 | 0.0030 | 0.3587 | — | 0.0003 |
| rs10511905   | 9   | G  | 0.22 | Additive | 0.0006 ($+)$ | 0.0087 | 0.1388 | 0.3149 | — | 0.0003 |
| rs518350     | 22  | T  | 0.12 | Recessive | 0.0065 ($+)$ | 0.0010 | 0.4987 | 0.2900 | 0.3334 | 2.70E-04 |
| rs17701271   | 4   | A  | 0.22 | Recessive | 0.0006 ($+)$ | 0.0026 | 0.1371 | 0.8756 | 0.2031 | 0.0003 |
| rs6002674    | 22  | C  | 0.15 | Recessive | 0.0012 ($+)$ | 0.0040 | 0.9808 | 0.0310 | 0.5675 | 0.0027 |
| rs1419555    | 7   | T  | 0.37 | Additive | 0.0043 ($+)$ | 0.0006 | 0.7723 | 0.4670 | 0.4932 | 0.0068 |
| rs1423515    | 5   | A  | 0.05 | Additive | 0.0200 ($+)$ | 0.0040 | 0.4104 | 0.4710 | — | 0.0089 |
| rs1522113    | 4   | A  | 0.05 | CLOCK | 0.0144 ($+)$ | 0.0377 | 0.9260 | 0.0427 | 0.8633 | 0.0177 |

Abbreviations: ACRN, Asthma Clinical Research Network; AT, Asthma Trial; CAMP, Childhood Asthma Management Program; Chr, chromosome; CARE, Childhood Asthma Research and Education Network; GACRS, Genetics of Asthma in Costa Rica Study; GWAS, genome-wide association study; LOCCS, Leukotriene modifier or Corticosteroid or Corticosteroid Salmeterol; LODO, Effectiveness of Low Dose Theophylline as Add-on Treatment in Asthma; MA, minor allele; MAF, minor allele frequency in CAMP; SNP, single-nucleotide polymorphisms. Association results for 13 replicated SNPs ($P$-values $< 0.05$) from the primary replication phase only are shown, sorted by Liptak Combined $P$-values. The $P$-values presented for CAMP are two-sided whereas $P$-values for the replication populations are one-sided, based on the direction of association (denoted as ‘+$‘ or ‘−$‘) relative to the CAMP analysis.

*Lowest $P$-value is with the additive generalized linear model.

*Lowest $P$-value is with the recessive generalized linear model.

*Lowest $P$-value is with the additive longitudinal analysis.

*Lowest $P$-value is with the family-based association test (FBAT).

*Lowest $P$-value is with the recessive longitudinal analysis.
secondary replication populations: rs166330, rs166332, and rs17495520, rs6002674 and rs1522113. The latter marker (additive P-value = 0.014 and β = 3.23 in CAMP) is located in intron 8 of CLOCK and in perfect linkage disequilibrium (correlation coefficient (r²) of 1.0 in CAMP) with a non-synonymous variant (rs34897046; Serine208Cysteine (S208C)) in exon 9 of the same gene.29 The top 13 SNPs explain 23.8% of the overall genetic variance in BDR, based on the correlation coefficient for each analysis. This calculation assumed that the genetic contribution of each SNP is independent of the other genetic associations.

Analysis of microarray data from lymphoblastoid cell lines from a subset of CAMP subjects determined that the missense variant in CLOCK is associated with variable gene expression of both CLOCK (P-value = 0.05) and one of its downstream effectors, Period 2 gene (PER2, P-value = 0.003) (Supplementary Figure 2). Individuals with one mutant allele (CG genotype, n = 20) had greater expression of both CLOCK and PER2 compared with individuals without this minor allele (GG genotype, n = 94). The SNP rs9888229 in the COL22A1 locus on the other hand did not demonstrate any cis-regulatory effects; however, it is correlated with the expression of multiple other genes (trans-acting effects on gene expression). This includes another member of the G protein-coupled receptor superfamily (GPR110). The top five trans-effects of each of the 13 SNPs from Table 2 are shown in Supplementary Table 1. These results do not suggest a regulatory role for the intragenic SNP on gene expression (rs11252394). Although these associations with gene expression suggest functional effects of some of our associated polymorphisms, further investigation is necessary to validate their functional effects and the mechanism by which they might regulate BDR.

**DISCUSSION**

This manuscript describes a comprehensive GWAS of treatment response to β₂-agonists in asthmatics, which identifies novel pharmacogenetic loci associated with clinical response variability. Owing to the limited size of the asthma drug trial populations, which is common in pharmacogenetic investigations, we implemented a novel strategy to select SNPs for replication. Specifically, we prioritized SNPs by evaluating P-values from five different statistical models, thereby taking advantage of the longitudinal nature of the phenotypic data, the entire sample at randomization as well as the genotype data from the parents. SNPs with the lowest P-values (<0.05) across all five statistical models were judged to represent the most robust associations, followed by SNPs yielding P-values <0.05 in four of the five analyses. The latter were prioritized by FBAT P-values for replication analysis. A total of 1397 were successfully genotyped and tested for replication in three independent clinical trials. The top 13 replicated SNPs were subsequently tested for association with BDR in three secondary asthma populations (Table 2). Although only one intergenic SNP significantly replicated in the primary phase, six SNPs provided nominal P-values <0.05 in both the primary replication phase and in one or more of the secondary replication populations, including intragenic SNPs in the COL22A1 and CLOCK genes.

The use of five statistical models in our initial GWAS is an innovative approach for identifying genetic associations for BDR in asthma. As each statistical model has unique strengths and weaknesses, our rationale for ranking SNPs for replication based on P-values from all five models was to identify the most robust associations (that is, those most likely to replicate and represent true pharmacogenetic associations). For example, population-based tests are more powerful for detecting associations by including more individuals than the number of informative families used in the FBAT, but the former is more vulnerable to population stratification. Thus, FBAT allows us to confirm SNP associations that are not influenced by population stratification. In addition, we were able to take advantage of the longitudinal BDR data recorded at 11 time points over the 4-year clinical trial for a subset of our population to confirm associations that are repeatable within individuals over time. Moreover, we opted to include a recessive model because, although an additive genetic model can easily identify dominant transmissions, it does not identify recessive transmissions as easily. We believe that this novel approach reduced the likelihood of false-positive association signals.

The strongest association signal that significantly replicated in the primary replication phase, albeit not associated across the secondary replication populations, was an intergenic SNP rs11252394 (Liptak P-value = 1.98E-07). Denoting it being not proximal to a gene within 50 kb, a closer look at this genomic region revealed several excellent biological candidates within 2.5 mb, including protein kinase C θ (PRKCG), inter-leukin receptors (IL15RA and IL2RA) and Krüppel-like factor 6 (KLF6). All four genes have been previously reported to regulate pulmonary inflammation using *in-vitro* cellular and murine models. In fact, a PRKCG antagonist was investigated by Wyeth Research as a novel treatment for asthma given the role of this gene in airway inflammation and hyper-responsiveness.39-42 Inhibition of IL15RA and IL2RA in mice demonstrated decreased lung inflammation.33-34 Finally, blocking of KLF6 in vitro decreased transforming growth factor β (TGFβ) production that is correlated with airway remodeling and asthma development.43 Although rs11252394 is not known to regulate the expression of any of these genes, nor is it known to be in linkage disequilibrium with SNPs within these loci, further investigation is warranted to identify the causative variant, if any, in this genomic region that may underlie this association signal.

Another association signal that replicated, albeit only marginally, in the primary replication phase and across two of the secondary replication trials, was an intergenic SNP (rs9888229) in the COL22A1 gene. Little is known about this gene other than that it encodes a protein that acts as a cell adhesion ligand for skin epithelial cells and fibroblasts; further investigations are necessary to determine how genetic variants at this locus might influence BDR. Cis-expression quantitative trait locus analysis indicates that this SNP does not regulate expression of the COL22A1 transcript (P = 0.86). However, this SNP is significantly correlated with the expression of other multiple genes (Supplementary Table 1). This includes another member of the G protein-coupled receptor superfamily (GPR110), to which the β₂-adrenergic receptor also belongs, which is known to regulate smooth muscle contractions and relaxations.40 Multiple splice variants of this gene, like many other members of this large gene family, has been shown to be expressed at significantly higher levels in airway smooth muscle cells.47

Although the polymorphism in the CLOCK gene (rs1522113) was only marginally associated with BDR at randomization in CAMP using the additive model (P-value = 0.014) and nominally replicated in AT/LOCCS/LODO and CARE, it is an excellent biological candidate for regulatory asthma as it suggests that CLOCK expression and β₂-agonists affect the expression of circadian rhythm genes that regulate asthma symptoms. Embryonic fibroblast cells from mice homozygous for mutant CLOCK expressed circadian rhythm genes in a non-cyclic manner, a phenotype that was rescued by ectopic expression of CLOCK.48 DeBruyne et al.49 reported that the circadian rhythm in peripheral tissues such as the liver and lung are also regulated by CLOCK. CLOCK binds to the E-box enhancer located 5' of circadian genes such as the Periods (PER) 1, 2 and 3 to regulate their expression.40 β₂-agonists have also been shown to induce the expression of human period 1 (hPER1) gene in bronchial epithelial BEAS-2B cells.50 Furthermore, the administration of β₂-agonists, particularly long-acting, reduces nocturnal asthma.51,52 β₂-agonists

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have also been shown to regulate the expression of these circadian rhythm genes through the phosphorylation of cAMP-responsive element-binding (CREB) protein that bind to CRE S' of these genes. The role of the circadian rhythm in asthma is apparent in that the narrowing of the airways are more severe between midnight and early morning hours. In addition, nocturnal asthma exacerbations are commonly experienced between 0400 and 0800 hours, which may be the combined effect of the circadian clock and the diminishing effect of asthma medications throughout the night.

In addition to a genetic association between rs1522113 and BDR in asthma, we determined that this intronic SNP is in perfect linkage disequilibrium with a missense variant in exon 9 (rs348970456; S208C), which is predicted to result in the loss of a (Serine) phosphorylation site. This coding SNP is predicted to be ‘deleterious’ by SIFT (Sorting Intolerant From Tolerant) or ‘possibly damaging’ by PolyPhen2. Finally, analysis of microarray data from lymphoblastoid cell lines of CAMP subjects indicates a marginal association between the mutant allele (208C) and increased expression of CLOCK (P-value = 0.054), as well as increased expression of a downstream circadian rhythm gene Period 2 (PER2, P-value = 0.003) (Supplemental Figure 2). Although this suggests that the associated polymorphism in CLOCK may be functional, further experiments are necessary to investigate the regulatory potential of this variant in the CLOCK pathway and the mechanism by which it modulates BDR.

Although this manuscript represents a comprehensive GWAS of BDR response in asthmatics aimed at identifying the most robust genetic associations for replication in additional asthma trials, there were several limitations. First, our initial GWAS used the phenotype of acute response to a short-acting β2-agonist (BDR at randomization) that was taken in all CAMP probands at the start of the study as well as repeated measures of BDR in a subset of the CAMP probands who were randomized to β2-agonist as needed over the 4 years of the trial. For the replication cohorts, however, we only used BDR measured upon entry into the respective studies as our replication samples did not have longitudinal data. Therefore, our replication results may not identify BDR associations in asthma patients taking β2-agonist over long periods of time. Second, the mean pre-bronchodilator FEV1,pp values varied across asthma populations (Table 1). Specifically, the values for all childhood asthma trials (CAMP, CARE and GACRS) were noticeably higher than those of adult asthma trials (AT, LOCCS, LODO and ACRN), which was expected. However, pre-bronchodilator FEV1,pp was adjusted for in our definition of BDR as well in all statistical analyses, by including it as a covariate. Finally, baseline medications and recruitment criteria varied across some of the populations. For example, the LOCCS trial had completed a run-in period of 4–6 weeks during which they were administered an inhaled corticosteroid that might have improved their lung function, resulting in reduced BDR. Finally, all participants of the AT trial had a minimum BDR of ≥15%. We addressed these differences across our trial populations in the pooled analysis of AT/LOCCS/LODO by coding each trial differently. Some of the trials (CAMP, CARE, GACRS) had wash-out periods during which they were taken off their regular asthma therapies but were permitted to use rescue medications as needed. Others such as CARE and ACRN had no wash-out periods. Thus, differences in medical histories may have influenced BDR. However, we believe that these differences further demonstrate the generalizability of our association results.

Although the aim of this GWAS was to identify novel loci for BDR, we noted that this study does not replicate all of the prior associated SNPs. These results were expected as it is unusual for all of the candidate genes to be significant in any one replication population. For some of these previously associated loci, the genetic effect sizes were very modest, making these genetic variants more difficult to identify. Power simulations, based on our sample sizes (n = 403, 764 and 1048) and the number of statistical tests, estimated that we had sufficient power (>90%) to identify common SNPs (minor allele frequencies >0.1) with effect estimates of ≥3%. In addition, there was not always adequate linkage disequilibrium coverage for some of the SNPs that were previously identified at candidate genes. Therefore, it was difficult to assess these genetic associations in our CAMP samples. Specifically, additional variants were genotyped in earlier studies using custom platforms that were not included on the Illumina HapMap550K Beadchip array used for the current GWAS. For example, none of the previously associated SNPs in ARGI were tested in the current GWAS. In fact, only four of the dozen SNPs previously implicated in the remaining three genes (A2B52, CRHR2 and ACO1) were directly tested in our GWAS. However, these did not yield high ranking scores for replication because we had selected SNPs based on P-values across five different statistical models. Furthermore, some of the earlier studies had reported a haplotype effect that was not tested in our study. Finally, our GWAS did not replicate the findings of the previous BDR GWAS, which reported association with SPATS2. A major difference in the current study is the combined analysis of longitudinal BDR measures as well as family-based data in addition to BDR at randomization using five statistical models, whereas the previous GWAS applied only one test of BDR at randomization.

Using a novel genome-wide association analysis method for investigating BDR in asthma, we have identified several genetic loci for further investigation. Among these findings is an intragenic SNP, rs11252394, that is located near multiple genes previously correlated with lung inflammation, which, therefore, are potential regulators of asthma. Other potentially interesting associations that were marginally associated with our drug response phenotype across multiple trials were intronic SNPs within the COL22A1 and CLOCK loci. Although microarray data indicate potential cis- or trans-effects of these SNPs, further investigation is merited to determine their biological significance and potential roles in modulating BDR to β2-agonists.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by U01 HL58599 and P01 HL03069 from the National Heart, Lung and Blood Institute (NHLBI). We thank all families for their enthusiastic participation in the CAMP Genetics Ancillary Study and the CAMP investigators and research teams, who were supported by the NHLBI N01 HR16049. Additional support for this research came from NHLBI grants N01 HR16044, N01 HR16045, N01 HR16046, N01 HR16047, N01 HR16048, N01 HR16049, N01 HR16050, N01 HR16051 and N01 HR16052. All data collection from the CAMP Genetic Ancillary Study was conducted at the Channing Laboratory of the Brigham and Women’s Hospital under appropriate CAMP policies and human subject’s protections. The CAMP Genetics Ancillary Study is supported by U01 HL075419, U01 HL65899, P01 HL083069, R01 HL086601 and T32 HL7427 from the NHI/NHLBI. Collection of microarray data from immortalized lymphoblastoid cell lines of CAMP subjects was supported by K23 HG003983 and R01 HL092197 from the NIH/NHGRI. We acknowledge the American Lung Association (ALA) and the ALA’s Asthma Clinical Research Centers investigators and research teams for use of LOCCS and LODO data, with additional funding from HL071394 and HL074755 from the NHLBI, and Nemours Children’s Clinic. GlaxoSmithKline supported the conduct of the LOCCS Trial by an unrestricted grant to the ALA. We acknowledge Sepcor for the use of the Asthma Trial data. The Single-Nucleotide Polymorphism Health Association Asthma Resource Project (SHARP) was funded by grants from the NHLBI U01 HL51510, U01 HL51634, U01 HL51831, U01 HL51845, U01 HL51843, M01 RR00079 and M01 RR03186, and was carried out by researchers from the Asthma Clinical Research Network (ACRN), CAMP and Childhood Asthma Research and Education (CARE) Network. Details are available in the Online Repository and on the dbGaP (database of Genotypes and Phenotypes) website: www.ncbi.nlm.nih.gov/sites/entrez?db=gap. The GACRS was supported by HL04370 and HL66289 from the NIH.
