Published in final edited form as:

Pharmacogenet Genomics. 2010 June ; 20(6): 351–358. doi:10.1097/FPC.0b013e328337f992.

GSNO Reductase and β_2 Adrenergic Receptor Gene-gene Interaction: Bronchodilator Responsiveness to Albuterol

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Abstract

Background—Short-acting inhaled β_2 -agonists such as albuterol are used for bronchodilation and are the mainstay of asthma treatment worldwide. There is significant variation in bronchodilator responsiveness to albuterol not only between individuals but also across racial/ethnic groups. The β_2 -adrenergic receptor (β_2 AR) is the target for β_2 -agonist drugs. The enzyme S-nitrosoglutathione reductase (GSNOR), which regulates levels of the endogenous bronchodilator S-nitrosoglutathione, has been shown to modulate the response to β_2 -agonists.

Objective—We hypothesized that there are pharmacogenetic interactions between GSNOR and β_2 AR gene variants which are associated with variable response to albuterol.

Methods—We performed family-based analyses to test for association between GSNOR gene variants and asthma and related phenotypes in 609 Puerto Rican and Mexican families with asthma. In addition, we tested these subjects for pharmacogenetic interaction between GSNOR and β_2 AR gene variants and responsiveness to albuterol using linear regression. Cell transfection experiments were performed to test the potential effect of the GSNOR gene variants.

Results—Among Puerto Ricans, several GSNOR SNPs and a haplotype in the 3'UTR were significantly associated with increased risk for asthma and lower bronchodilator responsiveness ($p = 0.04$ to 0.007). The GSNOR risk haplotype affects expression of GSNOR mRNA and protein, suggesting a gain of function. Furthermore, gene-gene interaction analysis provided evidence of pharmacogenetic interaction between GSNOR and β_2 AR gene variants and the response to albuterol

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in Puerto Rican ($p = 0.03$), Mexican ($p = 0.15$) and combined Puerto Rican and Mexican asthmatics ($p = 0.003$). Specifically, GSNOR+17059* β_2 AR+46 genotype combinations (TG+GG*AG and TG+GG*GG) were associated with lower bronchodilator response.

Conclusion—Genotyping of GSNOR and β_2 AR genes may be a useful in identifying Latino subjects, who might benefit from adjuvant therapy for refractory asthma.

Keywords

Asthma; Bronchodilator responsiveness; GSNO Reductase; β_2 -Adrenergic Receptor; Latinos; Gene-gene interaction; Polymorphisms; Pharmacogenetics

Introduction

Asthma is a common but complex respiratory ailment caused by the interaction of genetic and environmental factors. Short-acting inhaled β_2 -agonists (albuterol) are the mainstay of asthma treatment worldwide.(1,2) Furthermore, among low income and minority populations in the U.S., albuterol is more likely to be the only medication used for asthma regardless of asthma severity.(3,4)

There is substantial variation in bronchodilator responsiveness between individuals and racial/ethnic groups.(2,5-7) Although a significant proportion of the individual and population variance is assumed to be due to environmental factors and measurement errors, previous investigators have demonstrated that there are strong genetic effects influential to bronchodilator responsiveness and these studies indicate that a clinically significant fraction of the total population variance is genetic in origin.(6,8) A search for genetic determinants of responsiveness to albuterol have yielded a large number of studies, most of which have focused on genetic polymorphisms within the β_2 - adrenergic receptor (β_2 AR) gene.(9,10) However, polymorphisms in the β_2 AR gene have been inconsistently associated with bronchodilatory response to β -agonists and with tachyphylaxis.(9,10) The most commonly studied functional polymorphism in the β_2 AR gene is SNP +A46G, which results in a change in amino acid sequence from arginine to glycine at codon 16. Although this SNP was initially associated with increased response to albuterol(11,12), it has subsequently also been associated with tachyphylaxis from long-term use of β_2 -agonists.(13) Furthermore, we and others have previously reported that pharmacogenetic associations with β_2 -agonists vary by population.(7,8,11,14,15) The fact that results vary within and between populations is suggestive of complex gene-environment and/or gene-gene interactions which may help to explain discordant results.

Nitric Oxide (NO) bioactivity *in vivo* is mediated primarily through the covalent modification of cysteine sulfur by NO to form S-nitrosothiols (SNOs). S-nitrosogluathione (GSNO), is the predominant SNO present in the airway lining fluid of the healthy lung and is a potent endogenous bronchodilator.(16) Children with severe asthma have decreased SNO content in the airway lining fluid(17) and adults with mild asthma undergoing segmental airway antigen challenge have evidence of accelerated SNO breakdown.(18) GSNO reductase (GSNOR) has been identified as a key regulator of SNO levels in the lung and airway reactivity in mice(19) and humans.(20) In a murine model of allergic asthma, deletion of the GSNOR gene resulted in increased lung SNO concentrations and protection from allergen challenge. The airways of GSNOR-deficient (GSNOR^{-/-}) mice were also protected from β_2 -adrenergic receptor desensitization under basal conditions, whereas supplementation of GSNO attenuated β_2 receptor desensitization in wild type mice. The molecular basis by which GSNO inhibits β_2 AR desensitization may relate to inhibition of G-protein coupled receptor kinases (GRKs), thereby preventing β_2 AR phosphorylation and downregulation.(21) More recently, we

observed increased GSNOR activity and low lung SNO content in subjects with mild asthma. The increased GSNOR activity correlated inversely with methacholine PC20 suggesting that GSNOR is a key regulator of airway hyperresponsiveness in asthma.(20)

Work done previously by Wu *et al.* and in our lab has shown associations between genetic variants in the GSNOR gene and asthma(22,23). Based upon the compelling results in mice (19) and human(20) and prior analyses of pharmacogenetic associations for β_2 AR in Latinos (11,14), we hypothesized that gene-gene interactions between genetic variants in the GSNOR and β_2 AR genes may modify bronchodilator responsiveness to albuterol. We investigated this hypothesis in Mexican and Puerto Rican asthma trios participating in the Genetics of Asthma in Latino Americans (GALA) study.(5,11) We chose to study these populations because U.S. vital statistics indicate that asthma prevalence, morbidity, and mortality are highest in Puerto Ricans and lowest in Mexicans.(24,25)

Methods

Subjects

A total of 609 Latino asthmatic trios (proband and their biological parents; total n = 1827) with complete spirometry data were analyzed in this study. Out of 609 trios, 273 were Mexican and 336 were Puerto Rican trios. Detailed recruitment criteria and subject characteristics are described elsewhere.(5,11) Briefly, asthmatic trios of Puerto Rican or Mexican ethnicity were recruited from the New York City, Puerto Rico, San Francisco Bay Area and Mexico City as part of the Genetics of Asthma in Latino Americans (GALA) study. Ethnicity was self-reported. In GALA, asthmatic subjects were enrolled only if both biological parents and all four biological grandparents were of the same ethnic backgrounds - Puerto Rican in New York and Puerto Rico and Mexican in San Francisco and Mexico City. Additional inclusion criteria for asthmatic patient were age between 8-40 years, a current physician diagnosis of asthma, and presence of asthma symptoms (wheezing, cough, or shortness of breath) over the last two years. The patient had to be clinically stable and without symptoms or upper respiratory tract infection for at least 6 weeks prior to participating in the study. Recruitment criteria were identical at each site. Total plasma IgE was measured in duplicate using Uni-Cap technology (Pharmacia, Kalamazoo, MI). Local Institutional Review Boards (IRBs) approved all the studies, and all subjects provided written, age-appropriate informed consent or assent.

Pulmonary Function Tests

Pulmonary function test results were expressed as a percentage of the predicted normal value using age-adjusted prediction equations from Hankinson.(26) A quantitative measure of bronchodilator responsiveness was calculated as ΔFEV_1 (change in forced expiratory volume in one second) which is the relative percent change in baseline FEV_1 (Pre- FEV_1) after administration of 180 μ g (2 puffs) for subjects < 16 years old, and 360 μ g (4 puffs) for subjects $\geq$ 16 years old for the GALA and SAGE studies. Details of the pulmonary function tests are described elsewhere.(5,11)

SNP Discovery

Sequencing of the GSNOR gene was performed on 48 unrelated asthmatic subjects, 24 of Mexican and 24 of Puerto Rican ethnicity (96 chromosomes). This number of subjects provides greater than 90% power to detect variants with an allele frequency of 5% or more. All exons, exon-intron boundaries, and two potential promoter regions (-711 bp to -113 bp and +2933 bp to +3531 bp predicted by the program GENSCAN; <http://genes.mit.edu/GENSCAN.html>) of the GSNOR gene were sequenced (GenBank Accession No. AY987960). Sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster

City, California) and ABI Prism 3700 sequencer (Applied Biosystems, Foster City, CA). The degree of linkage disequilibrium (LD) was estimated by using the r^2 statistic.(27)

Genotyping

The five GSNOR SNPs (+3207 in the potential promoter region and +16701, +16777, +17059 and +17250 in the 3'UTR region) were further selected for genotyping in the entire GALA cohort based on their allele frequency (minor allele frequency > 5%), location (exonic or in potential promoter region) and the haplotype structure of the GSNOR gene. All five SNPs were genotyped using the AcycloPrime-FP™ (PerkinElmer) method.(28) Detailed information for SNP genotyping are provided in the online supplement. Genetic variant +A46G in the β_2 AR gene, which corresponds to amino acid change from Arg to Gly at codon 16, was previously genotyped and AA genotype has been shown to be associated with bronchodilator responsiveness in Puerto Rican, but not in Mexican asthmatics.(11,14)

Statistical Analysis

Mendelian inconsistencies were identified using PEDCHECK.(29) A total of 3 families were excluded due to Mendelian inconsistencies (0.5%, 3/609). Families with Mendelian inconsistencies were excluded from further analysis including Hardy-Weinberg Equilibrium (HWE) and family-based association tests. The HWE was calculated by means of χ^2 goodness-of-fit tests, separately for Puerto Ricans and Mexicans. The HWE was calculated separately for parents and probands within Puerto Ricans and Mexicans. FBAT(30) and HaploFBAT (31) were used to perform transmission disequilibrium tests to assess the association between individual SNPs and haplotypes, respectively, with asthma and quantitative measures of asthma-related phenotypes in the GALA families. The minor homozygotes and heterozygotes were combined for the analysis as the minor allele frequency of most of the genotyped GSNOR SNP is low. The quantitative phenotypes analyzed include baseline lung function (measured as Pre-FEV₁, % of predicted) and bronchodilator responsiveness (measured as Δ FEV₁, relative % of predicted). Odds ratios (OR) and their 95% confidence intervals for associations between individual SNPs and asthma were estimated using the program UNPHASED.(32)

Gene-gene interactions for bronchodilator responsiveness were tested using non-parametric linear regression models. An interaction term (cross product of SNPs in the GSNOR and β_2 AR genes) was included in the regression model along with the main effects and confounding variables such as age, sex, Pre-FEV₁, asthma duration, log transformed body mass index (log₁₀ BMI), steroid use and regular or as needed bronchodilator use to test for association with Δ FEV₁. Genetic association analyses may be confounded by population stratification. Therefore, the interaction analyses were also adjusted for individual ancestral proportions estimated using 107 unlinked ancestry informative markers (AIMs) using the method described elsewhere.(33,34) In addition, the interaction analysis performed on combined Mexican and Puerto Rican asthmatics was also adjusted for Latino ethnicity. All analyses were performed using the statistical software package Stata/SE 9.0 (Stata Corp., College Station, TX).

Correction for Multiple Testing—The correction for multiple testing was performed using SNPSpD (an effective Bonferroni-type correction).(35) Each phenotype (asthma, Pre-FEV₁ and Δ FEV₁) in the present study was tested for 5 SNPs. There is significant LD between these SNPs and therefore the program SNPSpD calculated these 5 SNPs to be equivalent to 2 independent SNPs. Thus, the α for GSNOR association tests was set at 0.008 [0.05/(2*3)], where 2 is the number of independent SNPs and 3 is the number of phenotypes tested. Since Mexicans and Puerto Ricans are two separate populations and could have different risk alleles; we did not correct the analysis within populations for two tests. The association analyses were performed separately in all asthmatics and on those asthmatics that were using albuterol as their only asthma medication. However, the latter group is not an independent cohort, rather a

subset of all asthmatics. Therefore analyses within all asthmatics and the subgroup of asthmatics using albuterol as their only asthma medication were not corrected for two tests.

Constructs and Cell Transfections

The full length GSNOR 3'UTR was amplified from human cDNA. The resulting PCR products were cloned into both forward and reverse orientations in the pGL3 plasmid containing an SV40 promoter upstream of the firefly luciferase gene (Promega Corporation, Madison, WI). Site directed mutagenesis of the 3'UTR was performed using QuikChange® II XL Site Directed Mutagenesis Kit (Stratagene, CA) according to the manufacturer's instructions. Individual mutants mimicking the SNPs associated with asthma in our patient population were generated by substituting C+16701→T, A+17059→C and T+17250→C (data not shown). Another mutant was created containing the three individual mutants to mimic the GSNOR haplotype associated with asthma. The mutated fragments were amplified and ligated into the pGL3 promoter vector. The luciferase reporter gene constructs were transiently transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen; Carlsbad CA) according to the manufacturer's protocol. Specific substrates were used to measure the activities of the firefly and *Renilla* luciferase with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Firefly luminescence was normalized to *Renilla* luminescence and reported as relative luciferase units (RLU). Differences among cell transfection groups were evaluated using a student's t-test. A p value of < 0.05 was considered statistically significant. Details of the constructs and the cell transfection assays are provided in the online supplement.

Results

Characteristics of the Subjects

Demographic and clinical characteristics of all probands with asthma that were analyzed for this study are shown in Table 1. Although the GALA cohort has a total of 686 trios with asthma (probands and their biological parents; total n = 1827), this study only included 609 trios that had probands with complete spirometry data (Mexican: n = 273 trios, Puerto Rican: n = 336 trios). The median age of the Mexican and Puerto Rican asthmatic probands was 13 and 12 years, respectively. The median Pre-FEV₁ and ΔFEV₁ were 89% and 7.4%; 83% and 4.9% in Mexican and Puerto Rican asthma subjects, respectively.

Resequencing of the GSNOR Gene

A total of 31 SNPs were identified in the GSNOR gene by resequencing the exons, exon-intron boundaries and two potential promoter regions in 24 Mexican and 24 Puerto Rican asthmatics (see materials and methods and Table 1S in the online supplement). Only 13 of these 31 SNPs had an allele frequency greater than 5% in Mexicans and Puerto Ricans. For both Mexicans and Puerto Ricans, we observed tight LD between the SNPs in the GSNOR gene (see Figure 1S in the online supplement). Five GSNOR SNPs were selected for further genotyping in the GALA cohort based on a minor allele frequency > 5% in Mexicans or Puerto Ricans, location (exonic or in potential promoter region) and linkage disequilibrium pattern. These SNPs included: +3207 in the potential promoter region, +16701, +16777, +17059 and +17250 in the 3'UTR region.

Association between GSNOR Genetic Variants and Asthma

The genotyping call rates for all five SNPs were > 95% and all SNPs were in HWE in Mexican and Puerto Rican parents (p > 0.05). Among Puerto Ricans, single locus family-based analyses demonstrated that several GSNOR SNPs were significantly associated with increased risk for asthma (Table 2). Associated genetic variants included: CT +TT genotypes of SNP +3207 in the promoter region (p = 0.05) and genotypes GA+AA, TG+GG and AG+GG of SNPs +16701,

+17059 and 17250, respectively in the 3'UTR ($p = 0.04$ to 0.007), and the 5 SNP haplotype T.A.G.G.G. with a frequency of 10% ($p = 0.02$) (Table 2 and 3). Only the association between SNP +17059 and asthma remained significant after correction for multiple testing ($p = 0.007$, adjusted $\alpha = 0.008$). No association was found between GSNOR SNPs or haplotypes with Pre-FEV₁ and Δ FEV₁. However, since steroids can blunt the effect of GSNOR,(36) the subjects were stratified by steroid use and the analysis was repeated in those subjects who were using albuterol as their only asthma medication. Although we had a significant reduction in sample size ($n = 168$) and therefore loss of statistical power, we observed that several GSNOR SNPs were significantly associated with Δ FEV₁ ($p = 0.04$ to 0.01) in Puerto Rican subjects who were using albuterol as their only asthma medication before correction for multiple testing (Table 2). A marginal association was also observed between the five SNP haplotype and Δ FEV₁ ($p = 0.06$) in this subgroup analysis (Table 3). Among Mexicans we did not find any association between GSNOR SNPs and asthma or related phenotypes including Δ FEV₁ either in the analysis including all asthmatics or analysis stratified by steroid use.

GSNOR and β_2 AR Gene-Gene Interaction

We performed gene-gene interaction analysis between the genetic variants of the GSNOR and β_2 AR genes for association with bronchodilator responsiveness using linear regression models. To avoid multiple testing issues, we only analyzed the gene-gene interaction between GSNOR +17059 and β_2 AR +46 SNPs. We selected GSNOR +17059 SNP as it showed the most significant association with bronchodilator responsiveness in our single gene analysis of GSNOR and SNP +46 (Arg16Gly) is one of the two most extensively studied SNPs for the β_2 AR gene and is in strong LD with the other most studied β_2 AR SNP +79 (Gln27Glu).

An interaction term of cross product between GSNOR +17059 and β_2 AR +46 genotypes was generated to test gene-gene interaction between β_2 AR and GSNOR genes (Table 4). Specifically, we paired the subject's genotype of GSNOR +17059 with genotype of β_2 AR +46 to determine if the presence of risk genotype for both the genes in a subject modified the risk for asthma differently than each gene alone. The association between the interaction term and Δ FEV₁ was tested using linear regression models. The linear regression models also included the two main effects SNPs GSNOR +17059 and β_2 AR +46 and other confounding variables as listed in the methods section. A significant association was observed between the interaction term and Δ FEV₁ for Puerto Ricans ($p = 0.03$) and a borderline association for Mexicans ($p = 0.15$). The association between the interaction term and Δ FEV₁ became more significant in the combined analysis of Puerto Rican and Mexican asthmatic subjects ($p = 0.003$) suggesting that the direction of association is in the same direction in Mexicans and Puerto Ricans. Table 5 shows the mean Δ FEV₁ stratified by interaction term after adjusting for main effects and confounders in Puerto Rican, Mexican and Puerto Rican and Mexican combined asthmatic subjects. Puerto Rican and Mexican asthmatics subjects, who had the GSNOR +17059 TG +GG genotype and either the β_2 AR +46 AG or GG genotypes (interaction term 1 and 2 respectively), had significantly lower bronchodilator responsiveness (mean Δ FEV₁ relative % predicted = 7.53 and 3.9 respectively) compared to those subjects that had the GSNOR +17059 TT genotype and any of the β_2 AR +46 genotype or GSNOR +17059 TG or GG risk genotype and β_2 AR +46 AA genotype (interaction term 0, mean Δ FEV₁ relative % predicted = 11.15) (Table 5). These results suggest a gene-gene interaction between GSNOR and β_2 AR genes for bronchodilator drug responsiveness and that specific combinations of GSNOR and β_2 AR genotypes are associated with lower Δ FEV₁ in both Puerto Rican and Mexican asthmatics.

Functional Analysis of GSNOR Risk Haplotype

The 3'UTR of a gene is important for mRNA maturation and stability. Therefore, we reasoned that the GSNOR 3'UTR SNPs may affect expression levels of GSNOR mRNA and protein. To show that the GSNOR 3'UTR construct was functional, we cloned the 3'UTR into the PGL

plasmid in both forward and reverse orientation. Only the 3'UTR inserted in the forward orientation demonstrated luciferase activity in HEK cells (data not shown). Moreover, the luciferase reporter activity from the construct containing the risk haplotype of GSNOR 3'UTR, was significantly higher than that from the wild type haplotype (Figure 1). These data suggest that the risk haplotype of GSNOR 3'UTR is a gain of function polymorphism.

Discussion

In this study, we identified SNPs in the promoter and the 3'UTR region of the GSNOR gene that are associated with increased risk of asthma in Puerto Rican subjects, as well as gene-gene interactions between GSNOR and β_2 AR that predict responsiveness to albuterol in both Mexicans and Puerto Ricans. Our functional studies further suggest that the GSNOR 3' UTR risk haplotype may represent a gain of function mutation, consistent with our discovery of increased GSNOR activity in mild asthmatics.(20)

The results of association analysis between SNPs in the GSNOR gene and asthma and related phenotypes in our Puerto Rican subjects support the importance of GSNOR in asthma and responsiveness to albuterol. Also it is interesting to note that the impact of GSNOR on response to albuterol appears to be ameliorated by use of inhaled corticosteroids. These results are consistent with previous work published by our group and by that of Gaston and colleagues showing SNOs protect from β_2 agonist desensitization(21) and steroids decrease GSNOR protein expression(36) If decreased GSNOR expression results in decreased GSNOR activity, then steroid therapy may increase SNO bioavailability and decrease desensitization to β_2 agonists in asthma. We did not find an association between GSNOR genetic variants and IgE levels or emergency department visits (data not shown) in either Mexican or Puerto Rican asthmatics. This is in agreement with the murine model of allergic asthma in GSNOR deficient mice that shows uncoupling of airway hyperresponsiveness and airway inflammation. No differences in levels of interleukin 13, IgE, mucus cell hyperplasia or other markers of inflammation between wild type and GSNOR deficient mice were observed.(19)

Although only one of the associations (SNP +17059 and asthma) between SNPs in the GSNOR gene and asthma and response to albuterol remained significant after correction for multiple testing, we performed multiple tests based on strong *a priori* hypothesis that GSNOR may play a role in asthma and bronchodilator responsiveness based on published literature.(19,23) Therefore, it may not be appropriate to disregard prior information on the role of GSNOR in asthma by using overly conservative correction methods for multiple testing such as Bonferroni correction. Wu *et al* previously described an association between the GSNOR gene and asthma in Mexicans.(23) There may be several reasons why we did not find an association between GSNOR genetic variants and asthma in our Mexican subjects. The study by Wu *et al* included 532 Mexican case-parent asthma trios and therefore had higher statistical power compared to our study which only analyzed 273 Mexican trios. In addition, 4 out of 5 SNPs analyzed in the two studies are different due to differences in selection criteria for SNPs for genotyping. Four of the 5 SNPs genotyped by Wu *et al* are intronic whereas SNPs analyzed in our study are in the promoter and 3' UTR region of the GSNOR gene. The SNP +3207 in the promoter region that was genotyped by Wu *et al* and also in our study was not associated with asthma in Mexican subjects in either study.(23)

We have previously provided evidence for an ethnic-specific association of β_2 AR Gly allele at codon 16 with lower bronchodilator responsiveness in our Puerto Rican asthmatics, but not in Mexican asthmatics.(11,14) Herein, we found evidence of a pharmacogenetic gene-gene interaction between genetic variants of the GSNOR and β_2 AR genes, which results in lower responsiveness to albuterol in both Mexican and Puerto Rican asthmatics. The interaction between the genetic variants of GSNOR and β_2 AR genes is consistent with the emerging

molecular and mechanistic understanding that these genes share a common signal-transduction pathway which mediates bronchodilator responsiveness (i.e. β_2 AR signaling).(21) The evidence of gene-gene interaction between GSNOR and β_2 AR genes can help explain inconsistency in association of β_2 AR genetic variants with responsiveness to albuterol in previous studies. These results suggest that two-locus genotype analysis of GSNOR and β_2 AR may be a better predictor of responsiveness to albuterol than single locus analysis.

We studied gene-gene interaction between GSNOR and β_2 AR for association with acute bronchodilator responsiveness to albuterol. In our Mexican and Puerto Rican GALA cohort we did not study the effect of chronic use of short acting β_2 -agonists and therefore we could not assess the effect of tachyphylaxis. It has been shown in some studies that the β_2 AR +46 AA genotype (Arg/Arg at codon 16), which is associated with better acute response to bronchodilator, may result in poor long term response due to desensitization of β_2 AR (tachyphylaxis). In mice, tracheal rings from GSNOR knockout mice maintain relaxation upon repeated exposure to the bronchodilator isoproterenol, whereas wild type mice demonstrate a decrease in responsiveness to treatment.(19) Protection from desensitization appears to occur through S-nitrosylation of G-protein kinase 2 (GRK2) by SNO(21), which results in decreased GRK2-mediated downregulation of β_2 AR by its phosphorylation. Therefore it would be interesting to extend these gene-gene interaction analyses between GSNOR and β_2 AR to randomized clinical studies of long-term bronchodilator use.

Given the strength of the association in the Latino population, a specific two-locus genotype may indicate the need for individualized pharmacologic therapy with GSNOR inhibitors and β_2 AR agonists, or additional adjuvant therapy. Likewise, Salpeter *et. al.* suggested that African American asthmatics receive additional therapy above and beyond therapies recommended by National Asthma Guidelines.(37) Patients with the combined risk alleles at the GSNOR and β_2 AR loci may represent a subgroup of asthmatics that are at risk for worse asthma outcomes. Further studies need to be performed to determine if genotyping at these loci can be used to tailor pharmacologic therapy to this patient subset in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge the families and the patients for their participation. The authors would also like to thank the numerous health care providers and community clinics for their support and participation in the GALA Study. The authors would like to especially thank Jeffrey M. Drazen, M.D., Scott Weiss, M.D., Ed Silverman, M.D., Ph.D., Homer A. Boushey, M.D. and Jean G. Ford, M.D. for all of their effort towards the creation of the GALA Study.

Supported By: This work was supported by National Institutes of Health (HL078885, HL088133, U19 AI077439, ES015794), Flight Attendant Medical Research Institute (FAMRI), and the RWJ Amos Medical Faculty Development Award to EGB), HL086887 to LGQ, National Institute of Environmental Health Sciences 5U19-ES012496 and NHLBI R01HL96673-01 to JSS, American Thoracic Society “Breakthrough Opportunities in Lung Disease” (BOLD) Award and Tobacco-Related Disease Research Program New Investigator Award (15KT-0008) to SC, the Ernest S. Bazley Grant to PCA, and the Sandler Center for Basic Research in Asthma and the Sandler Family Supporting Foundation.

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Figure 1.

Luciferase activities in HEK293 transfected cells. HEK293 cells were transiently transfected with the GSNOR wild type (WT) and risk haplotype vectors. Cells were harvested 48 hours after transfection. Protein extracts from cells were analyzed in a luciferase assay. The increased luciferase activity of the GSNOR 3'UTR risk haplotype construct was statistically significant ($p < 0.009$ vs. wild type)*. Values are means $\pm$ standard error; $n = 6$ per group. (Relative luciferase units, RLU) reported were calculated by normalizing firefly luminescence to *Renilla* luminescence.

Table 1

Demographic and clinical characteristics of Puerto Rican and Mexican subjects with asthma from the GALA families.

	Puerto Ricans (n = 336)	Mexicans (n = 273)
Characteristic		
Age (yrs) *	12 (10:15)	13 (11:20)
Sex (% male)	56.7%	53.0%
BMI (kg/m ²) *	21.3 (17:26)	23.7 (20:28)
Mild Asthma (%)	32.6%	32.9%
Serum IgE (IU/mL) *#	254 (95:632)	246 (100:591)
Baseline spirometry		
Pre-FEV ₁ *	83 (74:93)	89 (77:100)
Pre-FEV ₁ < 80%	38%	30%
Bronchodilator responsiveness		
ΔFEV ₁ (relative % predicted) *	4.9 (0.6:10)	7.4 (4:13)

* Values are expressed as median (25th:75th percentile).

Serum IgE levels were missing for 33 Puerto Rican and 20 Mexican subjects.

Table 2

Association of the GSNOR SNPs and 5 SNP haplotypes with asthma and related phenotypes in Puerto Rican asthma families before correction for multiple testing. A stratified analysis was also performed for those subjects who were using albuterol as their only asthma medication.

	SNP Genotype (Allele Frequency, Allele)									
	+3207 CT+TT/CC (0.26, T)		+16701 GA+AA/GG (0.10, A)		+16777 TG+GG/TT (0.37, G)		+17059 TG+GG/TT (0.11, G)		+17250 AG+GG/AA (0.16, G)	
	p-value	OR (95% CI) or Mean (SD)	p-value	OR (95% CI) or Mean (SD)	p-value	OR (95% CI) or Mean (SD)	p-value	OR (95% CI) or Mean (SD)	p-value	OR (95% CI) or Mean (SD)
All Puerto Rican Subjects (n = 336)										
Asthma	0.05 (+)	1.17 (1.03 - 1.57)	0.04 (+)	1.35 (1.05 - 2.02)	0.42 (+)	1.08 (0.97 - 1.45)	0.007 (+)	1.41 (1.10 - 2.10)	0.01 (+)	1.40 (1.15 - 1.97)
Pre-FEV ₁	0.23 (-)	83.6 (17.4)/84.9 (16.4)	0.88 (-)	84.2 (19.6)/84.5 (16.3)	0.80 (+)	83.9 (17.9)/84.7 (16.2)	0.82 (+)	85.7 (21.5)/83.6 (15.6)	0.27 (+)	85.2 (20.1)/84.1 (15.6)
ΔFEV ₁	0.13 (-)	5.2 (12.9)/7.4 (10.2)	0.85 (+)	7.2 (14.2)/6.4 (13.2)	0.39 (-)	6.5 (15.0)/7.1 (10.6)	0.93 (+)	7.5 (14.4)/6.5 (13.0)	0.69 (-)	7.2 (13.3)/6.4 (13.4)
Puerto Rican Subjects using albuterol only (n = 168)										
Asthma	0.19 (+)	1.18 (0.93 - 1.78)	0.20 (+)	1.38 (0.89 - 2.52)	0.50 (+)	1.13 (0.90 - 1.70)	0.09 (+)	1.44 (0.94 - 2.54)	0.02 (+)	1.68 (1.15 - 2.78)
Pre-FEV ₁	0.96 (+)	88.1 (15.4)/85.9 (14.0)	0.24 (+)	92.5 (17.5)/85.2 (14.1)	0.90 (+)	86.8 (17.0)/86.2 (13.0)	0.11 (+)	93.3 (17.7)/84.4 (13.9)	0.17 (+)	91.8 (17.1)/84.5 (14.0)
ΔFEV ₁	0.01 (-)	3.4 (7.2)/7.3 (10.3)	0.03 (-)	2.9 (6.4)/7.1 (15.4)	0.49 (-)	6.4 (16.4)/6.7 (10.7)	0.01 (-)	2.9 (6.3)/7.4 (15.5)	0.01 (-)	3.9 (6.9)/7.3 (15.9)

“+” and “-” indicate direction of association. “+” indicates increased risk for asthma or higher value of the quantitative phenotypes Pre-FEV₁ and ΔFEV₁ whereas “-” indicates decrease risk for asthma and lower value of the quantitative phenotypes. p < 0.1 are shown in bold.

Table 3

Association of the GSNOR 5 SNP haplotypes with asthma and related phenotypes in Puerto Rican asthma families before correction for multiple testing. A stratified analysis was also performed for those subjects who were using albuterol as their only asthma medication.

	5 SNP Haplotype (Frequency)					p-value
	C.G.T.T.A (0.63)	T.G.G.T.A (0.14)	T.A.G.G.G (0.10)	C.G.G.T.A (0.06)	C.G.G.T.G (0.05)	
All Puerto Rican Subjects (n = 336)						
Asthma	0.67 (-)	0.71 (+)	0.02 (+)	0.38 (-)	0.89 (-)	
Pre-FEV ₁	0.50 (+)	0.26 (-)	0.74 (-)	0.64 (-)	0.55 (+)	
ΔFEV ₁	0.53 (+)	0.11 (-)	0.59 (+)	0.90 (+)	0.35 (-)	
Puerto Rican Subjects using albuterol only (n = 168)						
Asthma	0.73 (-)	0.65 (+)	0.17 (+)	0.21 (-)	0.32 (+)	
Pre-FEV ₁	0.76 (-)	0.19 (-)	0.10 (+)	0.56 (+)	0.44 (-)	
ΔFEV ₁	0.06 (+)	0.42 (-)	0.19 (-)	0.40 (-)	0.34 (-)	

“+” and “-” indicate direction of association. “+” indicates increased risk for asthma or higher value of the quantitative phenotypes Pre-FEV₁ and ΔFEV₁ whereas “-” indicates decrease risk for asthma and lower value of the quantitative phenotypes. p < 0.1 are shown in bold.

Table 4

The interaction term, the cross-product between GSNOR +17059 and β_2 AR +46 genotypes and its coding for gene-gene interaction analysis.

GSNOR +17059 (T/G) genotypes	GSNOR +17059 (T/G) genotype coding	β_2 AR +46 (A/G) genotypes	β_2 AR +46 (A/G) genotype coding	Interaction term GSNOR+17059* β_2 AR+46	Interaction term GSNOR +17059* β_2 AR+46 coding	No. of Subjects (Puerto Ricans and Mexicans combined)
TT	0	AA	0	TT*AA	0*0 = 0	90
TG+GG	1	AA	0	TG+GG*AA	1*0 = 0	18
TT	0	AG	1	TT*AG	0*1 = 0	193
TG+GG	1	AG	1	TG+GG*AG	1*1 = 1	43
TT	0	GG	2	TT*GG	0*2 = 0	136
TG+GG	1	GG	2	TG+GG*GG	1*2 = 2	32

Table 5

Mean ΔFEV_1 (relative % predicted) for each interaction term adjusted for two main effects and potential confounders including age, sex, Pre- FEV_1 , asthma duration, log transformed body mass index ($\log_{10}$ BMI), steroid use, regular or as needed bronchodilator use and individual ancestral proportions in Puerto Rican, Mexican and Mexican combined asthmatic subjects. The table also shows number of asthmatic subjects for each interaction term category (n), standard error (Std. error) for the mean, 95% confidence interval lower and upper bounds (lb, ub) and p-value for the association of interaction terms with ΔFEV_1 (interaction term "0" is the reference).

Interaction term *	Puerto Ricans						Mexicans						Puerto Ricans and Mexicans combined					
	n	Mean ΔFEV_1	Std. error	lb	ub	p-value	n	Mean ΔFEV_1	Std. error	lb	ub	p-value	n	Mean ΔFEV_1	Std. error	lb	ub	p-value
0	225	8.92	0.82	7.31	10.54	ref	212	11.15	0.66	9.85	11.88	ref	437	10.04	0.55	8.96	11.11	ref
1	33	4.60	1.59	1.48	7.73	0.009	10	7.53	2.27	3.06	11.30	0.15	43	5.29	1.33	2.67	7.90	0.002
2	22	0.28	3.53	-6.66	7.22	0.02	10	3.90	4.74	-5.44	12.13	0.12	32	0.54	2.87	-5.10	6.17	0.001

* Interaction term "0" corresponds to the genotype combinations TT*AA, TG+GG*AA, TT*AG or TT*GG; interaction term "1" corresponds to genotype combination TG+GG*AG; and interaction term "2" corresponds to genotype combination TG+GG*GG as calculated in Table 3.