

Functional variants of the sphingosine-1-phosphate receptor 1 gene associate with asthma susceptibility

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Background: The genetic mechanisms underlying asthma remain unclear. Increased permeability of the microvasculature is a feature of asthma, and the sphingosine-1-phosphate receptor (S1PR1) is an essential participant regulating lung vascular integrity and responses to lung inflammation.

Objective: We explored the contribution of polymorphisms in the *S1PR1* gene to asthma susceptibility.

Methods: A combination of gene resequencing for single nucleotide polymorphism (SNP) discovery, case-control association, functional evaluation of associated SNPs, and protein immunochemistry studies was used.

Results: Immunohistochemistry studies demonstrated significantly decreased S1PR1 protein expression in pulmonary vessels in lungs of asthmatic patients compared with those of nonasthmatic subjects ($P < .05$). Direct DNA sequencing of 27 multiethnic samples identified 39 *S1PR1* variants (18 novel SNPs). Association studies were performed based on genotyping results from cosmopolitan tagging SNPs in 3 case-control cohorts from Chicago and New York totaling 1,061 subjects (502 cases and 559 control subjects). The promoter SNP rs2038366 (–1557G/T) was found to be associated with asthma ($P = .03$) in European Americans. In African Americans an association was found for both asthma and severe asthma for intronic SNP rs3753194 (c.–164+170A/G; $P = .006$ and $P = .040$, respectively) and for promoter SNP rs59317557 (–532C/G) with severe asthma ($P = .028$). Consistent with predicted *in silico* functionality, alleles of the promoter SNPs rs2038366 (–1557G/T) and rs59317557

(–532C/G) influenced the activity of a luciferase *S1PR1* reporter vector in transfected endothelial cells exposed to growth factors (epidermal growth factor, platelet-derived growth factor, and vascular endothelial growth factor) known to be increased in asthmatic airways.

Conclusion: These data provide strong support for a role for *S1PR1* gene variants in asthma susceptibility and severity. (J Allergy Clin Immunol 2010;126:241–9.)

Key words: Asthma, sphingosine-1-phosphate receptor 1, single nucleotide polymorphism, promoter activity

Asthma is a chronic inflammatory lung disease characterized by airway inflammation, increased vascular permeability,¹ and subsequent airway remodeling.^{2–4} Recently, the influence of angiogenic factors, such as vascular endothelial growth factor (VEGF),⁵ on asthma pathobiology has emerged, with increased levels of VEGF observed in asthmatic airways,⁶ likely resulting in increased microvascular permeability, mucosal edema, and airway diameter narrowing, thereby amplifying the effect of airway smooth muscle contraction.⁷ Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) levels are also increased in asthmatic airways,^{8,9} mediating airway smooth muscle proliferation and asthma remodeling.^{10,11}

We previously demonstrated that another angiogenic factor,^{12,13} sphingosine-1-phosphate (S1P), a multifunctional lipid mediator, is essential for regulation of vascular integrity and restoration of vascular barrier function under inflammatory states.¹⁴ S1P rapidly stimulates vascular endothelial cells and smooth muscle cells through ligation of a G protein–coupled S1P receptor family, including S1P receptors 1 to 5. Stimulation of the sphingosine-1-phosphate receptor 1 (S1PR1), which is highly expressed in vascular endothelial cells,^{15,16} results in barrier stabilization or reversal of barrier disruption induced by edemagenic agents, events driven by robust Rac GTPase-mediated cytoskeletal rearrangement.¹⁴ In murine and canine models ligation of S1PR1 also attenuates lung vascular permeability induced by LPS, ischemia and reperfusion, excessive mechanical ventilation, and radiation.^{17–19} Disruption of the *S1PR1* gene (*S1pr1*^{–/–}) in mice resulted in embryonic lethality caused by massive vascular permeability and hemorrhage at embryonic days –12.5 to –14.5.^{20,21} Furthermore, heterozygous *S1pr1*^{+/-} or wild-type mice pretreated with an S1PR1 antagonist have reduced S1P-mediated barrier protection after LPS challenge.^{17,18,22} Mice engineered to selectively lack S1P in plasma displayed increased vascular leakage, which was associated with increased interendothelial cell gaps in venules, and the addition of an agonist for S1PR1 rescued the vascular integrity.²³

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Abbreviations used

AA:	African American
AIM:	Ancestry informative marker
EA:	European American
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
Egr2:	Early growth response gene 2
HWE:	Hardy-Weinberg equilibrium
OR:	Odds ratio
PDGF:	Platelet-derived growth factor
S1P:	Sphingosine-1-phosphate
S1PR1:	Sphingosine-1-phosphate receptor 1
<i>S1PR1</i> :	Gene encodes sphingosine-1-phosphate receptor
<i>S1pr1^{-/-}</i> :	Knockout of the <i>S1pr1</i> gene in mouse
SNP:	Single nucleotide polymorphism
tSNP:	Tagging single nucleotide polymorphism
VEGF:	Vascular endothelial growth factor

Direct roles for S1P and S1P receptors, such as S1PR1, in asthmatic patients are inconclusive. Increased levels of S1P have been identified in bronchoalveolar lavage fluid from allergic asthmatic patients after ragweed antigen challenge, suggesting that S1P might serve a potential proinflammatory function in asthmatic airways²⁴ and participate in airway hyper-responsiveness through modulation of airway smooth muscle cell function and autocrine mediator of activated mast cells.²⁵⁻²⁷ In contrast to these potentially detrimental effects of S1P, intra-tracheal administration of FTY720, a fungal derivative and S1P analogue, significantly suppresses Th2-dependent eosinophilic airway inflammation and bronchial hyperresponsiveness, the cardinal features of asthma.²⁸ Modulation of S1PR1 expression also regulates immune cell trafficking through transactivation; through internalization, ubiquitination, and subsequent degradation; or through interaction with the lymphoid cell-surface molecule CD69.²⁹

Given the complex mechanisms involved in S1P/S1PR1 participation in asthma and the emerging importance of barrier regulation in asthma pathobiology, we hypothesized that *S1PR1* genetic variants can modify asthma susceptibility and severity. Our results indicate *S1PR1* as a novel asthma candidate gene and an attractive target for future therapeutic strategies.

METHODS**Immunohistochemistry study**

Postmortem formalin-fixed, paraffin-embedded lung tissue sections (5 μ m) were obtained from 7 asthmatic patients (3 European Americans [EAs] and 4 African Americans [AAs]) and 7 nonasthmatic subjects (3 EAs and 4 AAs) involved in fatal motor vehicle accidents. S1PR1 protein expression was detected by using rabbit polyclonal antibodies against human S1PR1 (sc-25489; Santa Cruz Biotechnology, Santa Cruz, Calif) in 1:150 dilution by using an indirect immunoperoxidase method (EnVision System; DAKO, Carpinteria, Calif) and counterstained with hematoxylin and eosin. All slides were coded and scanned in blinded fashion. A minimum of 5 areas in the subepithelial region were analyzed, and the total S1PR1 content was determined per unit area (104 μ m²) by using a computerized image analysis system (ImageJ, National Institutes of Health).^{30,31} The quantity of endothelial S1PR1 expression was normalized by using the endothelial marker VE-cadherin detected with murine mAbs at 1:50 dilution (sc-9989, Santa Cruz).

Human *S1PR1* gene resequencing

DNA samples of 27 unrelated nonasthmatic subjects (14 AAs and 13 EAs) were sequenced to search for common variation in the *S1PR1* gene. DNA sequencing protocols and polymorphism identification were performed by Polymorphic DNA Technologies (Alameda, Calif). PCR primers were designed to amplify the entire gene and 2 kb of the predicted promoter (Genomatrix Software, Inc., Ann Arbor, Mich) and downstream regions of the gene. A list of the primer pairs used is provided in Table E1 (available in this article's Online Repository at www.jacionline.org). The genomic sequence NM_001400 was used as the reference sequence, and variation was reported according to established guidelines.³²

Study populations and demographics

A total of 110 EA and 192 AA unrelated asthma cases and 209 EA and 193 AA control subjects were collected in Chicago as part of the Collaborative Study on the Genetics of Asthma protocol.³³ Chicago control subjects were adults reporting a negative personal and first-degree family history for asthma and either at least 3 grandparents of African American ancestry or of European ancestry. They should be considered population-based control subjects because medical histories were not obtained and medical testing was not performed. A detailed clinical description of case samples has been reported previously.^{34,35} Additionally, samples from 200 asthmatic patients and 157 control subjects from central or west Harlem, New York (all self-reported as African Americans), were enrolled as part of population-based asthma epidemiology studies at Harlem Hospital Center for asthma exacerbations.³⁶ In New York samples affection status, both in cases and control subjects, was determined by qualified physicians using standard diagnostic criteria.³⁷ Severe asthma was determined by using the National Asthma Education and Prevention Program Guidelines (1997) based on nighttime symptoms, asthma exacerbations, and an FEV₁ of less than 60% of predicted value at any time. Protocols were approved by the institutional review board, and written informed consent was obtained from all participants.

Genotyping

Genotyping was conducted with the iPLEX Gold Platform according to the manufacturer's protocol (Sequenom, San Diego, Calif), except for the single nucleotide polymorphisms (SNPs) rs1411017, rs61780687, and rs3737577, which were genotyped with TaqMan allelic discrimination assays (Applied Biosystems, Foster City, Calif). Briefly, iPLEX assays were scanned by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and individual SNP genotype calls were automatically generated with Sequenom TYPER 3.4 software. TaqMan genotyping was performed with a 7900HT Fast Real-Time PCR System (Applied Biosystems), with automated calls generated by using the SDS software based on discriminating plots (95% confidence). Approximately 10% of the samples were genotyped by duplicate to monitor genotyping quality. Genotyping was blind to case-control status and the ethnic background of the sample.

Assessment of genomic ancestry

Samples collected in Chicago had been previously assessed for 30 highly informative microsatellites of individual ancestry to European, West African, and Native American/Asian populations^{38,39} selected from about 2,000 microsatellites genotyped in a previously described multiethnic cohort of 35 European Americans, 88 African Americans, 34 Chinese subjects, and 29 Mexican Americans.⁴⁰

All samples were genotyped for 96 ancestry informative markers (AIMs) selected for being highly divergent ($\delta > 0.6$) among European, West African, and Native American/Asian populations to estimate individual ancestries of New York participants.^{41,42} Table E2 (available in this article's Online Repository at www.jacionline.org) shows a list of the AIMs used and frequency divergences among reference populations used to derive the estimates of this study (60 Europeans and 131 West Africans). Kosoy et al⁴³ showed that subsets as small as 24 AIMs of this panel were useful

for ascertaining the origins of subjects from different continents among American populations.

Individual ancestries were estimated from multilocus genotype data by using the Bayesian Markov chain Monte Carlo method implemented in the program STRUCTURE 2.1.⁴⁴ STRUCTURE 2.1 was run under the admixture model by using prior population information and independent allele frequencies. The Markov chain Monte Carlo method with a predefined number of parental populations ranging from $K = 1$ to $K = 3$ and a burn-in length of 50,000 for 70,000 repetitions was used. Note that although both AIM sets used are discriminatory for European, West African, and Asian/Native American ancestry, the program was used to determine the number of subpopulations ($K = 2$ or $K = 3$) in the African American samples. The results showed that the model with the best likelihood included $K = 2$ subpopulations showing significant West African and European ancestral proportions in our case-control samples, which is congruent with previous observations.^{38,45,46} Summary statistics for the estimated European ancestry for all population groups is supplied in Table E3 (available in this article's Online Repository at www.jacionline.org).^{40,43}

Statistical analysis

The multiple-marker selection algorithm haplotype r^2 included in TagIT 3.03 software⁴⁷ was used to select a set of cosmopolitan tagging SNPs (tSNPs) under previously described criteria.³⁸ This algorithm maintains the tSNPs' prediction accuracy and increase tagging efficiency by selecting fewer tSNPs than other methods.⁴⁸ Departures from Hardy-Weinberg equilibrium (HWE) were tested by means of an efficient exact test.⁴⁹ All individual SNP associations with disease were assessed by using the Cochran-Armitage trend test with prespecified scores of 0, 1, and 2 as risk probabilities based on the copies of risk alleles in the genotypes (ie, assuming an additive model) and the empiric P value obtained by using 1,000 permutations. All comparisons were performed against control subjects. Both HWE and trend test statistics were computed by means of a custom script for STATISTICA (StatSoft, Inc, Tulsa, Okla). A false discovery rate was assessed by using QVALUE,⁵⁰ not considering the 2 conditions tested because of the nonindependent nature of both traits, to judge the significant SNP associations in the context of the multiple comparisons performed. SPSS 14.0 software (SPSS, Inc, Chicago, Ill) was used for multiple logistic regression analysis and to obtain the odds ratio (OR) and its 95% CI as measures of the strength of the effects. For Chicago samples, individual ancestry was the only covariate considered to adjust in logistic regression models because no further data were shared among control subjects and cases, although extensive phenotype characterization was performed for the latter.³³ For New York samples, age, sex, and smoking status, along with the estimates of ancestry, were used for the adjustments in logistic regression models. The Student t test was used for comparison of promoter activity and protein expression between different groups. Statistical significance was set at a P value of less than .05.

Subcloning, transfection, and luciferase activity assay

DNA fragments of the *S1PR1* gene containing the alleles rs2038366_G (–1557G) or rs59317557_C (–532C) were amplified by means of PCR with the human genomic DNA and modified subsequently by means of site-directed mutagenesis to generate rs2038366_T (–1557T) and rs59317557_G alleles (–532G), respectively. All amplicons were verified by means of direct sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and fused to a pGL3-basic reporter vector (Promega, Madison, Wis) containing a genetically engineered firefly luciferase gene without a promoter. Human pulmonary artery endothelial cells (Cambrex, Walkersville, Md) were cultured in EBM-2 complete medium at 37°C and 5% CO₂/95% air within passages 6 to 10, as described previously.^{14,51} Plasmid constructs were transfected into human pulmonary artery endothelial cells with FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, Ind) in Opti-MEM medium for 2 hours. A plasmid with the renilla luciferase gene (phRL-TK) was cotransfected with pGL3-fused constructs as a transfection efficacy control. Cells were harvested 48 hours after transfection and lysed in Passive Lysis Buffer. Luciferase activity was measured with Dual-

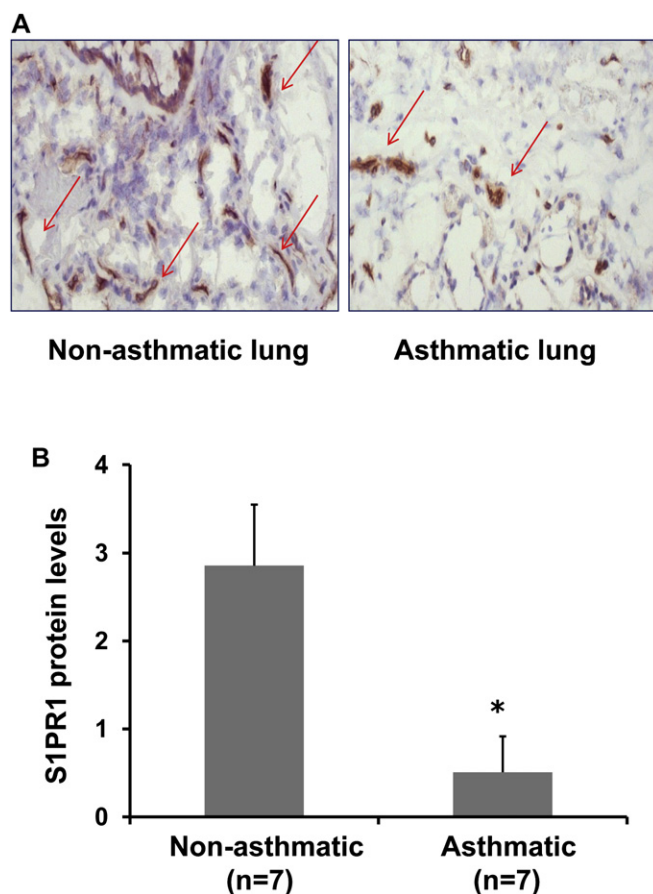


FIG 1. Levels of S1PR1 protein expression in pulmonary vessels detected by means of immunohistochemistry staining. **A**, Illustrative images of the frozen human lung sections from asthmatic and nonasthmatic subjects stained with anti-S1PR1 antibody. Arrows indicate S1PR1 protein staining in pulmonary arteries. **B**, Fold change (y axis) of S1PR1 protein level detected in nonasthmatic and asthmatic subjects depicted in this bar graph. * $P < .05$ versus nonasthmatic subjects.

Luciferase Assay Kits (Promega). The relative promoter activities were expressed as the ratio of firefly luciferase in pGL3 to renilla luciferase in phRL-TK (RLU) according to the Dual-Glo luciferase reporter assay protocols recommended by the manufacturer. Five independent transfections and duplicate luciferase assays were performed for each condition, along with pGL3-basic vector used as a control. The effects of specific growth factors (PDGF, 50 ng/mL; VEGF, 50 ng/mL; and EGF, 100 ng/mL) on luciferase activity assays were performed to assess the regulation of transcription factors (early growth response gene 2 [Egr2] or epidermal growth factor receptor [EGFR]) related to *S1PR1* promoter SNPs.

RESULTS

S1PR1 protein levels are decreased in the lung vasculature of asthmatic patients

Initial studies examined the tissue protein expression of S1PR1 in lungs from nonasthmatic and asthmatic subjects. Immunohistochemistry studies detected S1PR1 protein expression both in alveolar epithelium and lung vascular endothelium (Fig 1, A). In pulmonary arteries from asthmatic subjects, S1PR1 protein expression was significantly decreased when compared with that seen in nonasthmatic subjects ($P < .05$; Fig 1, B). The levels of S1PR1 protein were not significantly different between AA and EA samples within the small disease groups (data not shown).

TABLE 1. Summary of variations (tSNPs shown in boldface) found by resequencing the *S1PR1* gene and 2kb flanking sequences in African American (AA) and European Americans (EA)

SNP no.	Position*	Location (effect)**	rs no.	Flanking (5')	Flanking (3')	MAF# (AA)	MAF# (EA)	Val†
1	101473084	-1959G>A§	-	GAAAATGAGT	GTTTAAAAAG	0.00	0.04	-
2	101473486	-1557G>T§	rs2038366	-	-	0.14	0.23	‡
3	101473532	-1511G>T§	rs17123892	-	-	0.05	0.00	‡
4	101473700	-1343A>C§	-	CTGTTCGATGA	TGCTTTAAAA	0.04	0.04	-
5	101474020	-1023T>C§	rs12407294	-	-	0.14	0.23	‡
6	101474069	-974T>C§	rs33967958	-	-	0.39	0.42	‡
7	101474427	-616C>T§	-	ACAGGAACCT	CCGGTTCATT	0.11	0.00	-
8	101474511	-532C>G§	rs59317557	-	-	0.39	0.42	‡
9	101474558	-485G>C§	-	TCGCGCGAAA	TGGCTCGGGG	0.04	0.00	-
10	101475134	c.-273G>C	rs4987248	-	-	0.04	0.08	‡
11	101475136	c.-271delG	rs3835397	-	-	0.25	0.38	‡
12	101475176	c.-231A>G	rs3765684	-	-	0.04	0.08	‡
13	101475297	c.-164+54G>A	-	GGTTGTGGTA	GGCTTAGGTG	0.04	0.00	-
14	101475412	c.-164+169G>A	-	AGCCCATCTC	ATCAGATTGT	0.04	0.00	-
15	101475413	c.-164+170A>G	rs3753194	-	-	0.04	0.08	‡
16	101475837	c.-164+594T>C	rs1411017	-	-	0.32	0.19	‡
17	101475939	c.-164+696G>C	-	TTTTGCAATC	AAATTAGAAA	0.04	0.00	-
18	101476041	c.-164+798T>C	rs17100952	-	-	0.07	0.00	‡
19	101476067	c.-164+824T>C	-	ACGAAACTTC	GGGATTTGCT	0.04	0.00	-
20	101476144	c.-163-822delG	-	GGGGTGGGGG	TGGGGTGGGG	0.07	0.00	-
21	101476159	c.-163-807G>T	rs56236417	-	-	0.11	0.00	‡
22	101476236	c.-163-730A>T	rs61780687	-	-	0.14	0.23	‡
23	101476497	c.-163-469G>A	-	TCCGAGGAGA	ACGTGGGCTG	0.04	0.00	-
24	101477081	c.-48G>A	-	TCTAGCGTTC	TCTGGAGTAG	0.04	0.00	-
25	101477084	c.-45T>C	rs3737578	-	-	0.04	0.15	‡
26	101477120	c.-9G>T	rs3737577	-	-	0.07	0.27	‡
27	101477160	c.32C>A (p.Ala11Asp)	-	CTGGTCAAGG	CCACCGCAGC	0.04	0.00	-
28	101477165	c.37C>G (p.Arg13Gly)	rs41287280	-	-	0.00	0.04	‡
29	101478834	c.*557T>C	-	CGATCATCTA	AGCAAATAGG	0.00	0.08	-
30	101478994	c.*717C>A	rs55872049	-	-	0.07	0.00	‡
31	101479030	c.*753C>T	-	CCATTGAAGC	GAAATCTGCA	0.04	0.00	-
32	101479328	c.*1051T>C	-	AGTCATTTTA	TGGATTTTTC	0.04	0.00	-
33	101479565	c.*1288dupT	-	TTGTTTTTTT	AAAAAGAATA	0.07	0.00	-
34	101479714	*53T>C¥	rs12090529	-	-	0.25	0.00	‡
35	101479716	*55T>A¥	-	TACTGGATTG	GTAGCTCTTT	0.04	0.00	-
36	101479862	*201A>G¥	-	TTAACACTGA	GAGTAGTAAA	0.04	0.00	-
37	101481168	*1507delA¥	rs35079261	-	-	0.14	0.08	‡
38	101481340	*1679T>C¥	rs12409786	-	-	0.04	0.15	‡
39	101481435	*1774T>A¥	rs1358733	-	-	0.18	0.23	‡

*Position in chromosome 1 according to NCBI build 36.

**Reference sequence is NM_001400.3.

#Minor allele frequency.

†Validation status: frequency available, ‡; unknown or by 2-hit as in dbSNP build 129, †.

§Distance from first nucleotide of NM_001400.3.

¥Distance from last nucleotide of NM_001400.3.

Identification of novel *S1PR1* polymorphisms

Resequencing of the entire *S1PR1* gene (except for a 307-bp repetitive stretch ~1,700-bp intronic region) and 2 kb of the upstream and downstream regulatory regions (a total of 8,774 bp per sample) identified 39 variant sites (35 single-base changes and 4 indels), with 18 variants not previously identified (Table I). Among the novel variants, 2 resided in the proximal promoter region (−616C/T and −485G/C), and 1 variant predicted a non-synonymous amino acid change in the coding region (Ala11Asp). A total of 10 variants (thereafter cosmopolitan tSNPs, Table I) were sufficient to provide an adequate coverage of common *S1PR1* gene variation in both EAs and AAs and were used to genotype case-control samples, as described in the Methods section.

Polymorphisms of *S1PR1* are associated with asthma in 3 case-control samples

Table II shows the minor allele frequency of genotyped *S1PR1* tSNPs by population group. Duplicate genotypes did not demonstrate discrepancies with the original genotypes (0% discordance rate). Details of HWE results can be found in Table E4 (available in this article's Online Repository at www.jacionline.org). We noted that SNP 29 showed strong departures from HWE in both EAs and AAs from Chicago and was monomorphic in New York AAs. Thus this SNP was not considered for further analyses. SNP rs17123892 was monomorphic in EAs. Samples with a genotyping completion rate of less than 80% were also removed. Final analyses were conducted for 8 tSNPs (exclusion of SNP29 and

TABLE II. Minor allele frequency for the *S1PR1* tSNPs genotyped

tSNP	Chicago samples						New York samples		
	European Americans (EAs)			African Americans (AAs)			African Americans (AAs)		
	Control subjects	Asthmatic patients	Patients with severe asthma	Control subjects	Asthmatic patients	Patients with severe asthma	Control subjects	Asthmatic patients	Patients with severe asthma
rs2038366	0.32	0.42	0.44	0.26	0.30	0.30	0.28	0.29	0.18
rs17123892	0.00	0.00	0.00	0.06	0.07	0.07	0.08	0.06	0.03
rs59317557	0.39	0.40	0.35	0.43	0.46	0.44	0.45	0.39	0.25
rs3753194	0.08	0.06	0.12	0.07	0.03	0.03	0.04	0.06	0.05
rs1411017	0.05	0.04	0.04	0.34	0.35	0.32	0.35	0.32	0.33
rs61780687	0.35	0.40	0.48	0.10	0.09	0.09	0.08	0.06	0.05
rs3737577	0.34	0.30	0.37	0.06	0.06	0.07	0.03	0.05	0.11
SNP 29	0.01	0.01	0.02	<0.01	<0.01	0.02	0.00	0.00	0.00
rs12090529	0.04	0.04	0.04	0.28	0.25	0.27	0.28	0.33	0.25
rs1358733	0.18	0.20	0.24	0.25	0.20	0.20	0.27	0.23	0.23

rs17123892) in 104 EA cases (27 with severe asthma) and 197 EA control subjects and for 9 tSNPs (exclusion of SNP29) in 184 AA cases (103 with severe asthma) and 191 AA control subjects from Chicago and 195 AA cases (20 with severe asthma) and 139 AA control subjects from New York (Fig 2).

In EAs from Chicago, the promoter SNP rs2038366 (–1557G/T) was associated with asthma susceptibility ($P = .013$, q value = 0.10; Fig 2, A). The association remained significant after adjusting for ancestry in a logistic regression model (adjusted risk per allele T was as follows: OR, 1.48; 95% CI, 1.03–2.14; $P = .036$). None of the tSNPs resulted in significant association with severe asthma in EAs. Asthma susceptibility in AAs from Chicago was associated with the intronic SNP rs3753194 (c.–164+170A/G; $P = .005$, q value = 0.045; Fig 2, B) and remained significant after adjusting for ancestry in a logistic regression model (adjusted risk per allele G was as follows: OR, 3.00; 95% CI, 1.37–6.60; $P = .006$). Moreover, this SNP was also significantly associated with severe asthma ($P = .027$, q value = 0.24; Fig 2, B) after adjusting for ancestry (OR, 3.03; 95% CI, 1.12–8.18; $P = .028$). In AAs from New York, the intronic SNP rs61780687 (c.–163–730A/T) showed significant association with asthma susceptibility ($P = .043$, q value = 0.39; Fig 2, C), although it was not significant after adjusting for ancestry (OR, 1.58; 95% CI, 0.88–2.85; $P = .124$). However, a promoter SNP, rs59317557 (–532C/G), was identified to be significantly associated with severe asthma ($P = .027$, q value = 0.24; Fig 2, C), and association remained significant after adjusting for ancestry (adjusted risk per allele C was as follows: OR, 2.53; 95% CI, 1.10–5.80; $P = .028$) and for all available covariates (OR, 2.72; 95% CI, 1.00–7.41; $P = .050$).

***S1PR1* promoter SNPs –1557G/T and –532C/G reside within predicted binding sites for transcription factors and potentially alter growth factor-induced *S1PR1* promoter activity**

To explore the functional regulatory mechanism or mechanisms of the associated *S1PR1* promoter SNPs, we performed transcription factor motif analysis to search for transcriptional factor binding sites that could be potentially interrupted by these variants. *In silico* analysis revealed that EGFR binding sites, encompassing the flanking sequences –1562 to –1552, are potentially interrupted by SNP –1557T, possibly resulting in loss

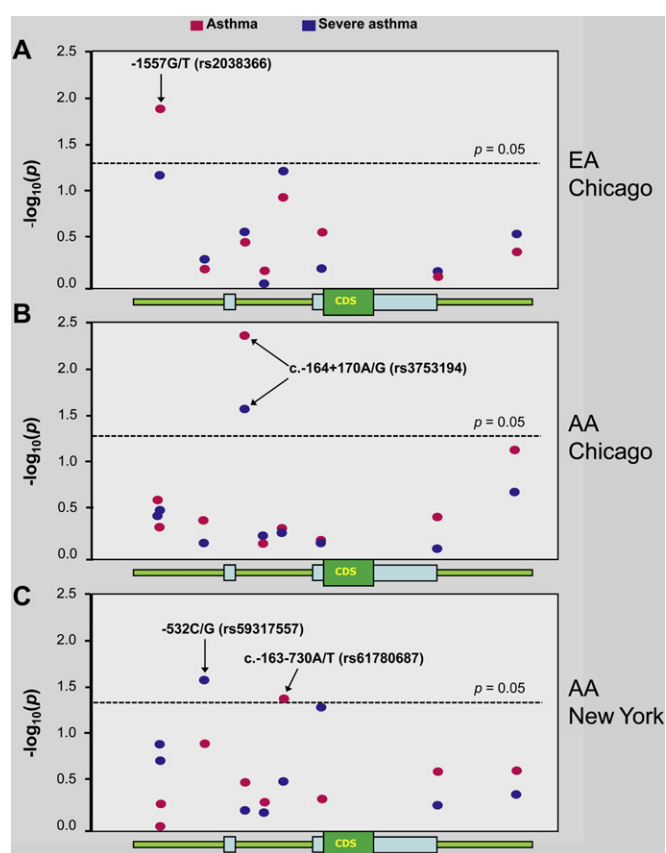


FIG 2. Plots of association P values of tested *S1PR1* tSNPs with asthma and severe asthma in European Americans from Chicago (A), African Americans from Chicago (B), and African Americans from New York (C). The dashed line represents a P value of .05. The *S1PR1* gene structure indicating the relative position of tested tSNPs for association is depicted at the bottom. CDS, Region of coding sequence.

of EGFR binding efficiency compared with the common allele –1557G. Similarly, the –532G allele potentially interrupts Egr2 binding to *S1PR1* promoter compared with the –532C allele (Fig 3, A). We next used a luciferase activity assay to assess the allelic effects of SNPs –1557G/T and –532C/G on *S1PR1* promoter activity. Under basal conditions (no stimulation), the activity of the *S1PR1* promoter with –1557T was significantly

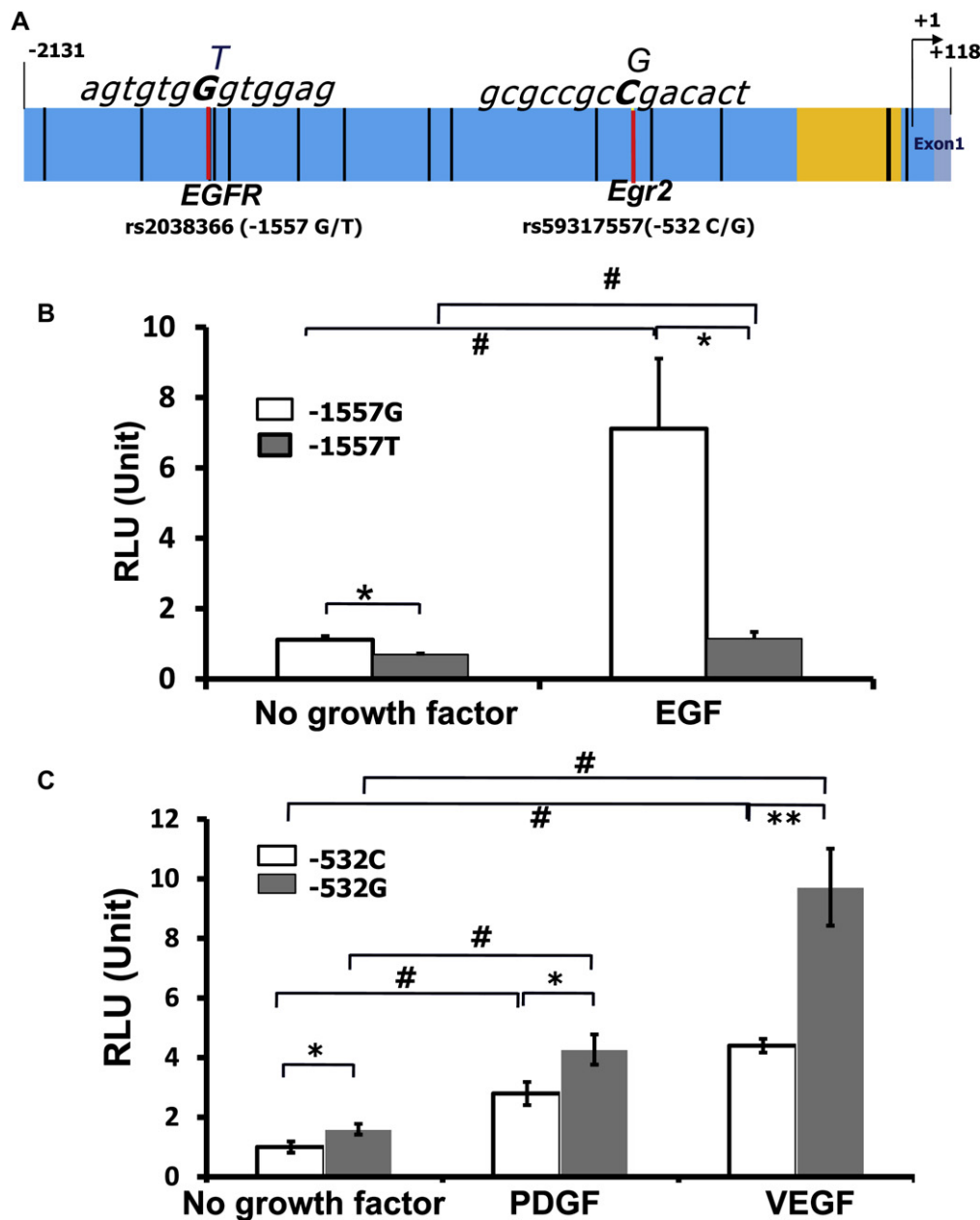


FIG 3. **A**, *In silico* analysis and promoter activity assay revealed a putative EGFR and Egr2 transcription factor binding site at positions -1557 (rs2038366) and -532 (rs59317557). **B**, Effect of the -1557G/T SNP on *S1PR1* promoter activity under basal conditions (no stimulation) and on EGF (100 ng/mL) stimulation. * $P < .05$ versus -1557G. # $P < .05$ versus no EGF. **C**, Effect of the -532C/G SNP on *S1PR1* promoter activity under basal conditions (no stimulation) and on PDGF (50 ng/mL) or VEGF (50 ng/mL) stimulation. * $P < .05$ versus -532G. ** $P < .01$ versus -532G. # $P < .05$ versus no PDGF or VEGF.

decreased compared with that of -1557G ($P < .05$; Fig 3, B, left panel). However, the luciferase activity of -532G was significantly increased compared with that of -532C ($P < .05$) under basal conditions (Fig 3, C, left panel). These data confirmed that variation of promoter SNPs at -1557T and -532G altered *S1PR1* gene expression. Next we tested whether variation at -1557 affected *S1PR1* gene transcription in response to EGF stimulation. Luciferase activity in endothelial cells transfected with either -1557G or T constructs followed by exposure of 100 ng/mL EGF for 2 hours resulted in significantly increased

reporter activity compared with that seen without EGF stimulation ($P < .05$). However, activity of the promoter containing -1557T exhibited a significantly attenuated response to EGF compared with -1557G ($P < .05$; Fig 3, B, right panel).

Finally, we assessed how another variation at -532 affected *S1PR1* gene transcription in response to growth factor activation of the binding Egr2 site. Growth factors PDGF and VEGF (50 ng/mL, 2 hours) significantly increased luciferase activity driven by both -532C and -532G compared with activity without growth factor stimulation ($P < .05$). However, growth

factor-mediated increases were significantly more pronounced for the *SIPR1* promoter containing the –532G allele compared with the –532C allele ($P < .01$; Fig 3, C).

DISCUSSION

We examined the role of S1PR1 in asthma, leveraging prior studies that revealed its ligand, S1P, a multifunctional lipid mediator and an angiogenic factor, to regulate key pathophysiologic features of asthma, including airway hyperresponsiveness, airway remodeling, and vascular permeability. S1PR1 is a dominant S1P receptor in many tissues and an important regulator that, in addition to its role in lymphocyte trafficking, regulates vascular permeability by endothelial integrity,¹⁵ regeneration,^{12,21} vasodilation,⁵² and migration.⁵³ Our immunohistochemistry studies demonstrated that S1PR1 protein expression in pulmonary arteries significantly decreased in lungs of asthmatic patients, suggesting that asthma development is significantly associated with decreased S1PR1 levels in endothelium. Subsequently, we explored the functional contribution of *SIPR1* polymorphisms to asthma susceptibility and severity through a combination of *SIPR1* gene resequencing for SNP discovery, case-control association studies, and luciferase assays. We identified *SIPR1* variants to be associated with asthma or severe asthma across unique case-control samples.

A strength of this study is that we performed an initial description of the common gene variation in a multiethnic panel of samples before selecting a cosmopolitan set of tSNPs to allow adequate coverage of other common untyped variants for the association analysis. More importantly, an additional strength is that gene association was found across 3 independent case-control samples totaling 1,061 subjects (502 cases and 559 control subjects). Moreover, we formally assessed the risk of false SNP associations in the context of the multiple comparisons and tested whether the association results might be affected by population stratification. The latter was performed by genotyping highly informative AIMs and estimating proportions of European and West African descent to be used in logistic regression modeling. Congruently, 2 of the associated SNPs demonstrated an effect in *in vitro* endothelial cell luciferase assays, providing strong functional evidence for the significant role of the *SIPR1* gene in asthma. It is worth noting that although associated SNPs are not in linkage equilibrium, these SNPs show poor correlation across European and African American samples (maximum $r^2 = 0.22$). Thus in addition to the strong evidence involving S1PR1 in asthma, our data provide support that *SIPR1* gene variants associate with asthma susceptibility. Genetic heterogeneity, environmental heterogeneity, or both; variability in phenotype definitions; interactions of an untested causal variant with the associated SNPs⁵⁴; small effects sizes; or inappropriate power could explain the failure to replicate findings at individual SNPs. In support of this latter possibility, although none of the individual SNP associations achieved statistical significance in all case-control samples, the direction of the effect for asthma susceptibility was consistent for one of the functional SNPs, rs2038366 (–1557G/T), across all 3 samples studied (OR_{GT} of 1.11 [95% CI, 0.84–1.49] and OR_{TT} of 1.68 [95% CI, 1.08–2.61], $P = .045$, Mantel-Haenszel test).

Our study has several limitations that need to be recognized, including that Chicago control subjects not clinically characterized for asthma might lead to a reduction in power. However, it is

well known that the consequences of misclassification of cases as control subjects are modest for a trait such as asthma (power reduction average, <4%).^{55,56} We used a tagging SNP approach after deep gene resequencing to study the association of the *SIPR1* gene with asthma. However, it is unclear whether the associations observed are due to a few or all of these SNPs or to other nearby variants within or flanking the gene. We designed this study to explore the association of common variants in the region and not to analyze the effect of rare variants for which the study is underpowered. Thus considering that resequencing of a few dozen samples is not sufficient for revealing all variations of the gene, it is likely that other variations within or outside the explored region might also contribute to disease susceptibility because no single SNP contributed to asthma in all populations. Conversely, such heterogeneity has been previously shown for a polymorphism firmly associated with asthma in strong susceptibility candidates, such as the *IL4RA* gene, encoding the α chain of IL-4 and IL-13 receptors.^{34,57}

We demonstrated functional differences for 2 asthma-associated SNPs. However, it remains unclear how the associated variants affect the S1PR1 activity *in vivo* and how such functional differences affect asthma susceptibility. To further explore the regulatory mechanisms of these promoter SNPs, we searched transcriptional factor binding sites potentially being interrupted by these variants and revealed EGFR and Egr2 sites for SNPs –1557 and –532, respectively. EGF mediates airway smooth muscle proliferation and asthma remodeling,⁵⁸ and S1P signaling plays an important role in EGF-mediated cell migration.⁵⁹ Recent studies further showed that receptor transactivation between EGFR and S1PR1 receptors engages an additional mechanism involving heparin-bound epidermal growth factor-like growth factor (HB-EGF) shedding from the plasma membrane by metalloproteinases.⁶⁰ The *SIPR1* promoter SNP –1557T demonstrated less affinity to EGF stimulation (75% reduction), suggesting that *SIPR1* transcription is regulated in response to EGF in endothelium. In contrast, the Egr2 transcriptional factor binding site acts as a transcriptional suppressor on activation by several growth factors, resulting in decreased gene expression of genes such as phosphatase 2A (*PP2A*).⁶¹ PDGF and VEGF are important factors in asthma and other inflammatory lung diseases^{5,7,10} and significantly increase levels of endogenous S1PR1 in endothelial cells.^{62,63} VEGF treatment of vascular endothelium markedly upregulates S1PR1 expression and enhances S1P-mediated signaling pathways leading to endothelial nitric oxide synthase activation.⁶⁴ Our data indicate that whereas *SIPR1* promoters with –532C and –532G alleles were responsive to PDGF and VEGF exposure, the –532G allele markedly and significantly influenced responses to PDGF and VEGF to a much greater degree than –532C, suggesting that variant –532G results in enhanced *SIPR1* transcription in response to growth factors, potentially leading to an improved integrity of the endothelial barrier or improved resolution of established vascular permeability. Because VEGF is well recognized to directly increase vascular permeability and asthma and PDGF worsens asthma symptoms, the increased *SIPR1* transcription could potentially counter effects of VEGF and PDGF on endothelial barrier dysfunction and severity of asthma.^{2,65,66} These effects are consistent with the –532C allele conferring an increased risk for asthma.

In summary, although the genetic mechanisms underlying chronic inflammatory lung syndromes such as asthma remain incompletely understood, our studies demonstrated that protein

expression of S1PR1, the endothelial dominant receptor for SIP and an essential participant in regulation of vascular integrity and angiogenesis, is decreased in asthmatic patients and that *S1PR1* variants constitute risk factors for asthma. Thus in agreement with the evidence supporting the benefits of asthma treatment with drugs targeting endothelial barrier enhancement, these data suggest that microvascular endothelium and S1PR1 might serve as potential targets for amelioration of asthma pathobiology.

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TABLE E1. Primers used for polymorphism discovery in the *S1PR1* gene

Amplicon	Nest forward primer (5'→3')*	Nest reverse primer (5'→3')	Size†	Boost forward primer (5'→3')	Boost reverse primer (5'→3')	Size†
A1	TTTATTGACTGATAGCGTTTGAGC	GAGGCCTCAAATCTCATTACTAGC	858	NA	NA	
A2	GTACAGGGTGTGTCTGCCAAT	CTACTGTATAAATCACTGCCCTCG	974	NA	NA	
A3	CTCAGCACACCGATCCTCCTA	AATAGAGATGAGAACAATCCCAGC	1132	NA	NA	
P1	ATTAGTACTGCCAGGAGCA	CCAGGGCAAGTAAATTTAAAGGTAT	527	TGGAGCGTGGGTAATGGCT	GACTGCCTTTTCTTTCTTCTTCTTA	622
P2	CAGTTTAATGTGAGTTTCTGAAGAG	ACCTCCTAATTGCCATGTGC	497	TAGCAATGCACAGGGAGC	CCCAGTTCAAAGATACTAGCA	538
P3	GCTTCACGGAGGCGC	GTTGGCTGTGGGTTGACAAA	497	ATTTACTTGCCCTGGCTTGG	TCAAAGGGGAAATGGGAATTACG	609
P4	TCTTTTCCGAGGAGAGACGT	CTGGAGAGGGCCTCG	510	CCCTCAGACTGGCAGTTG	ATCCAGGCTTTTGTGTAGCTTT	560
P5	ATAGAGTTTCTCCTCTCTTTTC	AATGCTGTTCTCCTTGTCG	527	CTTTGAGTGAATGTGGCCAT	GAACACCACCGAGGTCACT	566
P6	AAGGCTCTCTCGCCTCG	TCAATGGCGATGGCGAGGA	500	ATCGAACCACCCCTGAAGC	GGAGTTTCATTTTCAGCATTGTGAT	556
P7	AATTCACCGACCCATGTAC	TCCGAGTCCTGACCAAGG	471	TTGCTGACCATTTGGAAAACCAA	TTGCGGAACGTCAGGCG	518
P8	TAATCAGCGCCTGCTGGGT	ATCCGGATGAAGGCCCGA	487	GGAGCAATAACTTCCGCCT	CAGAGTCTCCGCTCGG	549
P9	TAATTATCGTCTGAGCGTCT	TCCTCCCTGGCAGCAGT	478	GCTGGCGCTGCTCAAG	AGGCTGTTCTGATTCTCCC	543
P10	GCAGCAAATCGGACAATTCC	ACTTGGGTTTGAAACTAAAGTGAAG	499	TCGCCGGCATGGAATTCAG	CAGAAAGTGACACACTCACT	537
P11	AACAATGCACTGGGAAGGGT	TCAACCAAATTCCAAAAACGGATC	499	GTGGGTAGAGTTAGTTCCTG	GCTTCAATGGATATGAAATGGTAAC	589
P12	TGCCCTGTACATCCCACAC	CAACAAAATACAACCATGTTGTTGC	560	TGTGCACTTCTGCTTCTTTAG	CCAATAAAATGACTTCTGCTGAAAC	645
P13	TAAATGATATTAGCCAGGATCCTTG	GATCCACAAAAGTCAGAAACCTATT	547	CATAAGGAAGCCCACTTTATCTAA	AATGCTCTTCACAAAGGGATCTTT	643
P14	CACCCTCTGTGCCCTTAA	TTACTAGCCAATGTGGTATTCAG	537	CGTGTTAACACCATTTGAATGTGTAT	GGTTTTGAATGATGATTGAGTCTC	727
P15	GATTGTGTAGCTCTTTGGAATCAA	TAGCTGCTTCACTGATACTGTATA	532	ATCCCTTTGTGAAGAGCATTCTTA	TCAGCCCACATTTTACAGCTA	600
P16	CACATTGGCTAGTAAGTAGAATTGT	ACATCTACTCTATTAGGTGAGGT	652	GATAAAAACCAACCTTCTGAAT	CACAGTTAACCTTACCCAGAAA	703
P17	CACTTTGCAAGCATCACAGA	TCATGAATCACTACTGTGCTTATGA	508	CCTTCAATTCATAGTATCTGAAAAGC	GCCAGCCACCTTATCCCAT	607
P18	TCCAAAGACAAAAGGCTGGAAA	CTCATTTCTACTTCTGAGAATACAC	506	CTGAAGGAAATGCCAAAAATGC	GTTTCACTCCTCTTGAAAAACACTA	620
P19	GATTAGATGTACTTTCTTCTGGAA	AAGGCAACACCTCATTTCTGA	556	TGTGACCACATTATCCAGTGAT	CTATGATTGTGCCACTGCATA	610
P20	CCTGGACAATACTTGACATATTCTT	CTCCCTCAAGGTATTTATAACCTAT	521	AAGAGGAGTGAAACAGATCCT	CTCTGATAGTCTCTACAAAAGTTTC	583

*Polymorphism discovery for amplicons P1 through P20 was performed by Polymorphic DNA Technologies. A “boost” amplification reaction was performed in a first round, and this product served as a template for the “nested” amplification and sequencing by using the nest primers.

†Amplification product in base pairs.

TABLE E3. Estimated European ancestry of case-control samples based on AIMs

	Chicago samples*				New York samples†	
	European Americans		African Americans		African Americans	
	Control subjects	Asthmatic patients	Control subjects	Asthmatic patients	Control subjects	Asthmatic patients
Mean	98.4%	99.8%	23.7%	24.9%	18.5%	16.0%
Median	100.0%	100.0%	21.5%	22.9%	14.7%	14.2%
Interquartile range	99.9% to 100.0%	99.9% to 100.0%	15.2% to 32.3%	16.5% to 32.0%	8.8% to 26.7%	7.6% to 20.8%

*Using 30 highly informative microsatellites of individual ancestry to European, African, and Asian populations.⁴⁰

†Using 96 SNPs highly divergent among European, African, and Native American populations.⁴³

TABLE E4. HWE *P* values for the *S1PR1* tSNPs genotyped

tSNP	Chicago samples						New York samples		
	European Americans			African Americans			African Americans		
	Control subjects	Asthmatic patients	Patients with severe asthma	Control subjects	Asthmatic patients	Patients with severe asthma	Control subjects	Asthmatic patients	Patients with severe asthma
rs2038366	.515	.839	1.000	.703	.469	.211	.537	.008	.468
rs17123892	NA	NA	NA	1.000	.562	1.000	.558	.560	1.000
rs59317557	.067	1.000	1.000	.074	.282	.394	.607	.364	.260
rs3753194	.613	1.000	1.000	.604	1.000	1.000	1.000	.152	1.000
rs1411017	1.000	.134	1.000	.747	.097	.486	1.000	.869	.607
rs61780687	.639	.839	.700	.097	.644	.143	.140	1.000	1.000
rs3737577	.205	1.000	1.000	.475	.010	.050	1.000	.044	.160
SNP 29	.001	.000	1.000	1.000	.000	.000	NA	NA	bNA
rs12090529	.272	1.000	1.000	.361	.847	1.000	1.000	1.000	.543
rs1358733	.218	.558	1.000	.563	.815	.539	.830	.689	1.000

P values significant after a Bonferroni adjustment for multiple comparisons ($\alpha = .0017$) are shown in boldface.

NA, SNP was found to be monomorphic in the sample.

Table E2. AIMS used in African American samples from New York

rs no.	Chromosome	Position (NCBI build 36.3)	Frequency European	Frequency West African	Delta (European/West African)
rs424436	1	8005265	0.99	0.95	0.04
rs7504	1	27110737	0.23	0.38	0.16
rs1931059	1	35138643	0.22	0.09	0.14
rs10908316	1	35267397	0.88	0.08	0.80
rs2791966	1	36087448	0.18	0.86	0.68
rs710232	1	41975741	0.96	0.25	0.70
rs596985	1	64049623	0.98	0.24	0.74
rs855833	1	64130020	0.92	0.11	0.81
rs17035850	1	116833545	0.97	0.15	0.82
rs6695965	1	145526715	0.86	0.12	0.74
rs2274533	1	146662406	0.23	0.48	0.25
rs2814778	1	157441307	0.99	0.00	0.99
rs12074150	1	171883530	1.00	0.35	0.64
rs2065160	1	203057600	0.87	0.54	0.33
rs6604611	1	214721874	0.10	0.87	0.77
rs6698938	1	228051979	0.99	0.25	0.74
rs2502342	1	241137090	0.06	0.73	0.66
rs883399	2	9551551	0.60	0.38	0.22
rs300152	2	17850164	0.19	0.24	0.05
rs11124405	2	35041515	0.93	0.14	0.79
rs13385952	2	41384902	0.84	0.06	0.78
rs1881244	2	73498597	0.07	0.87	0.80
rs12714168	2	86184858	0.86	0.11	0.75
rs6576989	2	96888826	0.90	0.09	0.82
rs260714	2	108928927	0.13	0.83	0.70
rs951954	2	109816794	0.01	0.79	0.78
rs901304	2	163124608	0.78	0.15	0.63
rs6748661	2	195391085	0.18	0.88	0.70
rs11713766	3	373089	0.02	0.83	0.81
rs2470644	3	5751857	0.92	0.22	0.70
rs2197896	3	30007534	0.95	0.12	0.83
rs9311121	3	35903067	0.05	0.87	0.82
rs13069719	3	71589235	0.20	0.23	0.03
rs2660769	3	87163186	0.72	0.01	0.71
rs12489482	3	106062098	0.15	0.86	0.71
rs6437783	3	109655507	0.83	0.72	0.11
rs11714866	3	111550592	0.91	0.20	0.71

rs6772085	3	120067253	0.93	0.19	0.74
rs2165139	3	140697160	0.89	0.96	0.07
rs6439896	3	141347043	0.97	0.34	0.63
rs1439013	3	154064681	0.92	0.25	0.67
rs9290363	3	170477801	0.08	0.88	0.80
rs10032047	4	63410428	0.04	0.94	0.90
rs7689609	4	72302238	0.84	0.00	0.84
rs7687935	4	82284590	0.80	0.20	0.60
rs7662047	4	103310753	0.03	0.89	0.86
rs7657799	4	105594872	0.97	0.21	0.76
rs13108157	4	151750213	0.12	0.94	0.82
rs6829588	4	165441421	0.17	0.81	0.65
rs2332031	4	171979533	0.88	0.12	0.76
rs814597	5	10521929	0.12	0.17	0.05
rs463240	5	25880903	0.05	0.78	0.73
rs35395	5	33984346	0.06	0.78	0.72
rs16891982	5	33987450	0.09	1.00	0.91
rs10059859	5	59267064	0.14	0.81	0.67
rs6894171	5	72798481	0.06	0.88	0.82
rs1443985	5	119453406	0.87	0.13	0.74
rs4513684	5	147632278	0.98	0.21	0.77
rs1551765	5	153156771	0.17	0.29	0.12
rs567442	5	176185891	0.93	0.13	0.80
rs6909271	6	143379	0.17	0.91	0.74
rs6459548	6	17590296	0.01	0.79	0.78
rs1341567	6	76684896	0.98	0.19	0.79
rs2497150	6	84903732	0.07	0.82	0.75
rs794672	6	95515038	0.12	0.95	0.83
rs218867	6	121440234	0.80	0.12	0.68
rs6930928	6	156647248	0.07	0.85	0.78
rs7810554	7	15107715	0.80	0.01	0.79
rs7784684	7	40134287	0.02	0.87	0.85
rs10264353	7	43287602	0.77	0.00	0.77
rs10257477	7	107491924	0.98	0.28	0.70
rs3094537	7	109341905	0.96	0.18	0.78
rs2021782	7	131785535	0.89	0.12	0.77
rs10954631	7	138190166	0.10	0.85	0.75
rs6601288	8	8980840	0.30	0.13	0.17
rs11778591	8	12764720	0.89	0.33	0.56
rs2439522	8	97602942	0.86	0.96	0.10

rs12347078	9	334508	0.95	0.12	0.83
rs4478653	9	21843221	0.59	0.18	0.41
rs587364	9	124800684	0.14	1.00	0.86
rs10748592	10	94868345	0.24	0.39	0.15
rs1572396	10	117315011	0.29	0.62	0.33
rs6485600	11	12216278	0.31	0.66	0.35
rs1638567	11	66881799	0.92	0.15	0.77
rs2458640	11	77713504	0.77	0.06	0.72
rs533571	11	100355412	0.30	0.16	0.14
rs4936512	11	119660546	0.79	0.69	0.10
rs1648180	11	127554612	0.30	0.44	0.14
rs2293048	12	116149208	0.13	0.56	0.43
rs1540979	13	93888693	0.85	0.96	0.12
rs730570	14	100212643	0.83	0.17	0.65
rs1129038	15	26030454	0.69	0.00	0.69
rs1426654	15	46213776	0.99	0.01	0.98
rs11073967	15	89366808	0.59	0.01	0.58
rs9937955	16	10858027	0.24	0.57	0.33
rs1557519	16	14158804	0.90	0.04	0.86