

ORIGINAL ARTICLE

## Role of Prostanoid DP Receptor Variants in Susceptibility to Asthma

Tsuyoshi Oguma, M.D., Lyle J. Palmer, Ph.D., Esra Birben, M.S.,  
Larry A. Sonna, M.D., Ph.D., Koichiro Asano, M.D., and Craig M. Lilly, M.D.

### ABSTRACT

#### BACKGROUND

From the Combined Program in Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston (T.O., E.B., L.A.S., C.M.L.); Western Australian Institute for Medical Research and University of Western Australia Centre for Medical Research, Perth (L.J.P.); U.S. Army Research Institute of Environmental Medicine, Natick, Mass. (L.A.S.); and the Cardiopulmonary Division, Department of Medicine, Keio University School of Medicine, Tokyo (K.A.). Address reprint requests to Dr. Lilly at the Pulmonary and Critical Care Division, Brigham and Women's Hospital, 75 Francis St., Thorn 826C, Boston, MA 02115, or at [clilly@partners.org](mailto:clilly@partners.org).

N Engl J Med 2004;351:1752-63.  
Copyright © 2004 Massachusetts Medical Society.

Previous genetic studies have associated the region of the human genome (14q22.1) containing the gene for the prostanoid DP receptor (*PTGDR*) with asthma. A study of a mouse model suggests that the receptor is required for the expression of the asthma phenotype. Our associations of asthma with functional genetic variants of *PTGDR* link these observations.

#### METHODS

We identified and evaluated combinations of genetic variants that influence *PTGDR* transcription for disease association in case-control studies of 518 white patients with asthma and 175 white controls and 80 black patients with asthma and 45 black controls.

#### RESULTS

We identified four novel and two previously reported single-nucleotide polymorphisms (SNPs) in *PTGDR* and its vicinity. These define four common three-SNP haplotypes, which vary in their ability to support transcription of *PTGDR* and have distinct DNA-binding-protein affinity profiles. Individual *PTGDR* SNPs were significantly associated with asthma in both populations. Specific *PTGDR* haplotypes were significantly associated with a diagnosis of asthma in a large case-control study of whites ( $P=0.002$ ); we confirmed these findings in a second population of blacks ( $P=0.01$ ). Multivariate analysis of the haplotype combinations (diplotypes) demonstrated that both whites (odds ratio, 0.55; 95 percent confidence interval, 0.38 to 0.80;  $P=0.002$ ) and blacks (odds ratio, 0.32; 95 percent confidence interval, 0.12 to 0.89;  $P=0.03$ ) who had at least one copy of the haplotype with a low transcriptional efficiency had a lower risk of asthma than subjects with no copies of the haplotype.

#### CONCLUSIONS

Our functional and genetic findings identify *PTGDR* as an asthma-susceptibility gene.

**A**STHMA MAY AFFECT AS MANY AS ONE in four urban children<sup>1</sup>; it is associated with substantial morbidity, mortality, and economic costs,<sup>2</sup> and despite intensive investigation, the factors that confer susceptibility are not well understood. Identifying the pathways that allow asthma to be expressed is fundamental to improving preventive strategies, developing diagnostic tools, and designing therapies.<sup>3</sup>

The prostanoid DP receptor gene (*PTGDR*) is located on chromosome 14q22.1 and encodes a heptahelical transmembrane G-protein-coupled receptor of 359 amino acids.<sup>4</sup> It is a candidate asthma gene because it is required for the expression of asthma<sup>5</sup> and because its genetic location has been linked to asthma and atopy.<sup>6-10</sup> The prostanoid DP receptor is required for the development of sensitization in a mouse model of asthma. The asthma phenotype does not develop in mice with a nonfunctional DP receptor.<sup>5</sup>

We sought out variants of *PTGDR*<sup>11</sup> and demonstrated that specific combinations of the variants had an effect on transcriptional efficiency. This observation provided the basis for our secondary hypothesis: that sequence variants in *PTGDR*, which limit its expression by reducing its transcriptional efficiency, are associated with reduced susceptibility to asthma.

METHODS

Screening for genetic variants of *PTGDR* was performed on DNA from 25 subjects (19 whites and 6 blacks) who met American Thoracic Society criteria for the diagnosis of asthma<sup>12</sup> and from 25 apparently healthy controls (20 whites and 5 blacks) with no history of asthma, atopy, or clinically significant medical illness.<sup>12</sup> Personal identifiers had been removed from all DNA samples. This number of subjects allowed us to detect variants that had a frequency of more than 5 percent. Ancestral origin was determined by self-report, and all subjects provided written informed consent. Genomic DNA for sequencing was extracted from lymphocytes immortalized with the use of an Epstein-Barr virus-based protocol and prepared as previously described.<sup>13</sup>

Genotyping used genomic DNA prepared from fresh whole blood collected from 518 white and 80 black patients with mild-to-moderate asthma on the basis of spirometric criteria<sup>14</sup> and from 175 white and 45 black controls without a history of asthma, atopy, or clinically significant medical illness. White

and black patients were recruited from 20 U.S. centers for a drug-treatment trial<sup>15</sup>; racially matched healthy controls were recruited from a U.S. Army population<sup>16</sup> (Table 1).

IDENTIFICATION OF SEQUENCE VARIANTS

Genomic DNA was screened for mutations in the protein-coding and 5'-flanking regions of *PTGDR* by single-strand conformational polymorphism analysis<sup>13</sup> with the use of oligonucleotide primers designed from the GenBank nucleotide sequence (accession number AC012407). Products of the polymerase chain reaction (PCR) whose electrophoretic mobility differed from that of the consensus sequence were reamplified, and the products were sequenced. Variants of the promoter and exon 1 sequences were identified by means of automated sequencing of DNA from 50 unique chromosomes, and the sequence of 30 unique chromosomes was determined for exon 2.

TRANSIENT TRANSFECTION ANALYSIS WITH PROMOTER REPORTER CONSTRUCTS

*PTGDR* reporter constructs of 1102 bp were created by means of PCR amplification of genomic DNA

Table 1. Demographic Characteristics of the Two Study Populations.\*

Characteristic	Controls	Patients	P Value
<b>Whites</b>			
No. of subjects	175	518	
Sex (no.)			0.006
Female	67	261	
Male	108	257	
Age (yr)	26.0±8.1	34.0±13.9	<0.001
Plasma total IgE (kU/liter)			<0.001
Geometric mean	35	140	
95% Confidence interval	26-46	120-160	
<b>Blacks</b>			
No. of subjects	45	80	
Sex (no.)			0.01
Female	28	31	
Male	17	49	
Age (yr)	23.5±5.3	33.0±11.8	<0.001
Plasma total IgE (kU/liter)			<0.001
Geometric mean	87	240	
95% Confidence interval	60-130	180-300	

\* Plus-minus values are means ±SD.

from homozygous subjects who had the alternative haplotypes with the use of primers that included the *KpnI* and *XhoI* recognition sites. Amplicons were cloned and ligated into a firefly luciferase vector and propagated according to standard techniques. Plasmid DNA was purified with the Qiagen plasmid purification system.

The effects of constructs (1.5  $\mu$ g) bearing *PTGDR* haplotypic variants were studied in transiently transfected A549 cells, grown to 80 percent confluence in six-well plates, and cotransfected with 75 ng of renilla luciferase vector with the use of Lipofectamine PLUS and Lipofectamine reagent (Invitrogen). Transfected cells were incubated for 24 hours and then placed in passive lysis buffer. Lysate firefly and renilla luciferase activities were assayed with the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was adjusted for renilla luciferase activity to control for differences in transfectional efficiency.

#### TRANSCRIPTION-FACTOR EXPRESSION CONSTRUCTS

A549 cells were transiently transfected with 500 ng of the CCAAT/enhancer binding protein  $\beta$  (C/EBP $\beta$ ) expression vector (kindly provided by Dr. S. Akira and Dr. M. Hoshino, Osaka University, Osaka, Japan) or 100 ng of a GATA-3 expression vector (kindly provided by Dr. L.C. Ho, Harvard School of Public Health, Boston), 1.5  $\mu$ g of firefly luciferase vector bearing the *PTGDR* haplotypic variant CCT, and 500 ng of  $\beta$ -galactosidase vector as previously described.<sup>17</sup> Schneider S2 cells grown in six-well plates with drosophila-SFM medium (Invitrogen) were transiently transfected with 500 ng of the Sp1 expression vector (pPACUSP1, kindly provided by Dr. E.S. Silverman, Harvard University, Boston), 1.5  $\mu$ g of pGL3-basic vector bearing *PTGDR* haplotypic variant CCT, and 500 ng of a  $\beta$ -galactosidase vector with the use of Cellfectin reagent (Invitrogen).<sup>18</sup> Lysate firefly activity was determined and  $\beta$ -galactosidase activity was measured by means of a  $\beta$ -galactosidase enzyme assay system according to the directions of the manufacturer. Firefly luciferase activity was adjusted for  $\beta$ -galactosidase activity to control for differences in transfectional efficiency.

#### ELECTROPHORETIC MOBILITY-SHIFT ASSAY

Nuclear extracts were prepared from human basophilic cell line KU812 (Japan Health Sciences Foundation) according to previously described tech-

niques.<sup>17</sup> Double-stranded oligonucleotides containing the following *PTGDR* promoter variants were synthesized: -197T, 5'CAGAGCGTCCC GCCTCTCAAAGAGGGGTGT; -197C, 5'CAGAGCGTCCGCCTCCCAAAGAGGGGTGT; -441C, 5'TCAAACACCAGCACCCTGCCCTCCTCTCAGGT; -441T, 5'TCAAACACCAGCACCATTGCCCTCCTCTCAGGT; -549T, 5'TGAGTTATCTTTACCTTTCCTTGA CTAGCTA; and -549C, 5'TGAGTTATCTTTACCTTTCCTTGA CTAGCTA. Oligonucleotides were radiolabeled with [ $\alpha$ -<sup>32</sup>P]deoxycytidine 5'-triphosphate with the Klenow fragment enzyme and purified by gel filtration. Binding reactions between protein and DNA were performed with 5 to 10  $\mu$ g of nuclear extract protein and 1  $\mu$ l of labeled oligonucleotide (50,000 cpm), 1  $\mu$ l of polynucleotide (polydeoxinosinic-deoxycytidylic acid) in 100 mM TRIS (pH 7.5), 10 mM EDTA, 10 mM dithiothreitol, and 50 percent glycerol in a total volume of 20  $\mu$ l. After incubation at room temperature for 30 minutes, protein-DNA complexes were resolved on a 7 percent nondenaturing acrylamide gel in a 0.5 $\times$  TRIS-borate-EDTA buffer (44.5 mM TRIS, 44.5 mM boric acid, and 1 mM EDTA) at room temperature and visualized by autoradiography. Supershift and cold-competition experiments were performed by preincubating nuclear extracts with 2  $\mu$ g of specific polyclonal antibodies or excess annealed cold oligonucleotide, respectively, for 10 minutes before the addition of the labeled probes.

#### DP RECEPTOR GENOTYPING AND HAPLO-TYPING

We determined the frequencies of the SNPs by means of restriction-fragment-length polymorphism (RFLP) analysis. We introduced a mutation (indicated by underlining) that created a distinguishing enzyme restriction site in the resulting PCR product by using modified primers when these sites were not present. We used the following oligonucleotide primer pairs: T-549C, sense, 5'CCACGGCAGATCACTTAAC, and antisense, 5'GGCTTCTACCTAGTTGTCTTGAATTAGCTAGTCAAGCCA; C-441T, sense, 5'CGAGTTCTTGGCCACCAGTTCAAACACCAGCACAA, and antisense, 5'GGAGCAGGCCAGTGAAGA; T-197C, sense, 5'ACTCGGCACCAGAGTCTGTC, and antisense, 5'AACCTCCTATCTAAACTCGCGGGTCAACCCCTCTTCG; and G+1044A, sense, 5'CGAGCCTTGCGATTCTATC, and antisense, 5'TCCTCAGCTTACCACAGAGTGA. PCR used *Taq* polymerase according to the instructions of the manufacturer, with buffers optimized for amplification specificity

and efficiency. Amplified DNA (10  $\mu$ l) was then digested with restriction enzymes (*TaqI* cuts T-197, *MfeI* cuts -441T, *BstNI* cuts -549C, and *BsrFI* cuts +1044A). Digested PCR products were electrophoresed on a 1.5 percent agarose gel containing ethidium bromide and visualized by means of ultraviolet transillumination. PCR product digests from subjects with sequences of the alternative genotypes were used to confirm the identity of the genotype in each assay.

Haplotype analysis of the *PTGDR* promoter region was determined by allele-specific PCR followed by RFLP analysis of the product. We amplified the genomic DNA using a variant-allele-specific primer at T-549C (sense, 5'CCAGACGTGAGTTA-TCTTTACGC, and antisense, 5'AACCTCCTATCTAACTCGCGGGTCACACCCTCTTCG) and digested the PCR fragments using a restriction enzyme (*TaqI* for T-197C and *BsrDI* for C-441T) that was specific for the wild-type allele. In each assay, controls of known sequence were correctly identified, and we confirmed the results by sequencing a subgroup of samples.

#### CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation assays were performed according to the method of Boyd et al.<sup>19</sup> with the use of a commercially available kit (Upstate) on nuclear extracts from KU812 cells and human peripheral-blood eosinophils isolated by density-gradient centrifugation and CD16-negative immunomagnetic selection from subjects bearing the forms of the *PTGDR* promoter in which transcription-factor binding was observed in electrophoretic mobility supershift assays. Control conditions excluded the antibody or genomic DNA or used an irrelevant antibody. Additional details are provided in the Supplementary Appendix (available with the full text of this article at [www.nejm.org](http://www.nejm.org)).

#### STATISTICAL ANALYSIS

The primary outcome variable of the association analyses was case-control status. The secondary outcome within the groups of patients was the total plasma IgE level. The principal explanatory variables were the individual *PTGDR* variants or their haplotypes. The SNP genotypes were divided into three classes and analyzed categorically, with the most common homozygous genotype for each SNP used as the reference category. Haplotypes were analyzed categorically, with the most common haplotype used as the reference category. Diplotypes were analyzed categorically relative to a baseline diplotype.

Each SNP locus was evaluated for Hardy-Weinberg equilibrium with the use of a contingency table of observed genotypic frequencies as compared with predicted genotypic frequencies according to a modified Markov-chain random-walk algorithm.<sup>20</sup> Pairwise linkage disequilibrium between each pair of SNP loci was analyzed by means of a likelihood-ratio test whose empirical distribution was obtained by mean of a permutation procedure.<sup>21</sup> Lewontin's disequilibrium coefficient  $D'$  was estimated from the directly determined (molecular) haplotype frequencies.

Bivariate analysis used analysis of variance to compare continuous outcomes across the levels of each genotype or haplotype and  $\chi^2$  tests or a hybrid approximation of Fisher's exact test<sup>22</sup> on contingency tables for comparisons of the distributions of categorical variables across alleles, genotypes, haplotypes, and diplotypes. For comparison of transcriptional activation, results of analysis of variance that were significant were followed by pairwise comparisons among haplotypes.

Generalized linear models (linear and logistic regression)<sup>23</sup> were used to model the effects of multiple covariates on the continuous and dichotomous outcomes, including an investigation of the need for interaction or polynomial terms. Sex and age were included as potential covariates in multivariate analyses.

Evidence of population stratification in our case-control samples was sought as previously described.<sup>15</sup> In brief, 29 unlinked SNPs separated by at least 20 MB were selected from the SNP consortium project (available at <http://snp.cshl.org/genome.shtml>) and genotyped in our white population, and 29 unlinked synonymous coding-region SNPs with a predicted frequency of at least 0.4 were genotyped in our black population as detailed in the Supplementary Appendix. The test statistics from each SNP were summed as  $\chi^2_{\text{S}} = \sum_{i \in L} \chi^2_i$ , where  $\chi^2_i$  is the  $\chi^2$  statistic computed at the  $i$ th marker locus and  $L$  is the set of unlinked marker loci typed. Under the null hypothesis,  $H_0$ ,  $\chi^2_{\text{S}}$  is  $\chi^2$  distributed, with the degrees of freedom equal to the total degrees of freedom of individual loci.<sup>24</sup>

We used S-Plus software version 6.1R3 (Mathsoft), Sib-Pair software version 0.99.9 (available at <http://www2.qimr.edu.au/davidD/>), and LogXact software version 4.1 (Cytel) to manage and analyze the data. P values were derived by empirical simulation where possible. A P value of less than 0.05 was considered to indicate statistical significance.

## RESULTS

**IDENTIFICATION OF SEQUENCE VARIANTS**

Sequencing revealed four novel variants of *PTGDR* (T-549C, C+367A, G+894A, and G+1044A) and two previously reported variants (T-197C and C-441T). We identified three variants in the 5'-flanking region (T-197C, C-441T, and T-549C), one missense variant (C+367A, Leu123Ile), and two synonymous coding-region variants (G+894A and G+1044A) (Fig. 1). The G+894A and C+367A alleles were rare, with a frequency of less than 1 percent in both populations, and were therefore not studied further.

**ASSOCIATION AMONG SNPs**

All four common SNPs were to some degree in linkage disequilibrium (Fig. 1 in the Supplementary Appendix). The synonymous coding region variant G+1044A was in almost complete linkage disequilibrium with the T-197C 5'-flanking region variant. Haplotypes were therefore defined by the three 5'-flanking region variants. Combinations of the promoter-region variants were used to define five unique three-SNP haplotypes, of which four were common (frequency, greater than 5 percent).

**TRANSIENT-TRANSFECTION ANALYSES**

We found significant differences in promoter activity among the haplotypic variants after transient-transfection analysis in A549 cells (Fig. 1B). The haplotype with sequence TCT (promoter positions, -549, -441, and -197) was associated with significantly lower reporter activity, and haplotype CCC was associated with significantly higher reporter activity, than haplotypes TTT and CCT: mean ( $\pm$ SE) CCT activity,  $3.2 \pm 0.07$  relative luciferase units (RLU); mean TTT activity,  $2.8 \pm 0.18$  RLU; mean TCT activity,  $2.4 \pm 0.07$  RLU; and mean CCC activity,  $5.2 \pm 0.07$  RLU, with respective values after the subtraction of background activity of  $1.1 \pm 0.07$  RLU,  $0.7 \pm 0.19$  RLU,  $0.4 \pm 0.07$  RLU, and  $3.1 \pm 0.07$  RLU (mean of five experiments;  $P < 0.001$  for all pairwise comparisons by analysis of variance except for the comparison of TCT with TTT, for which  $P < 0.05$ ).

**EFFECTS OF VARIANTS ON DNA-BINDING PROTEINS**

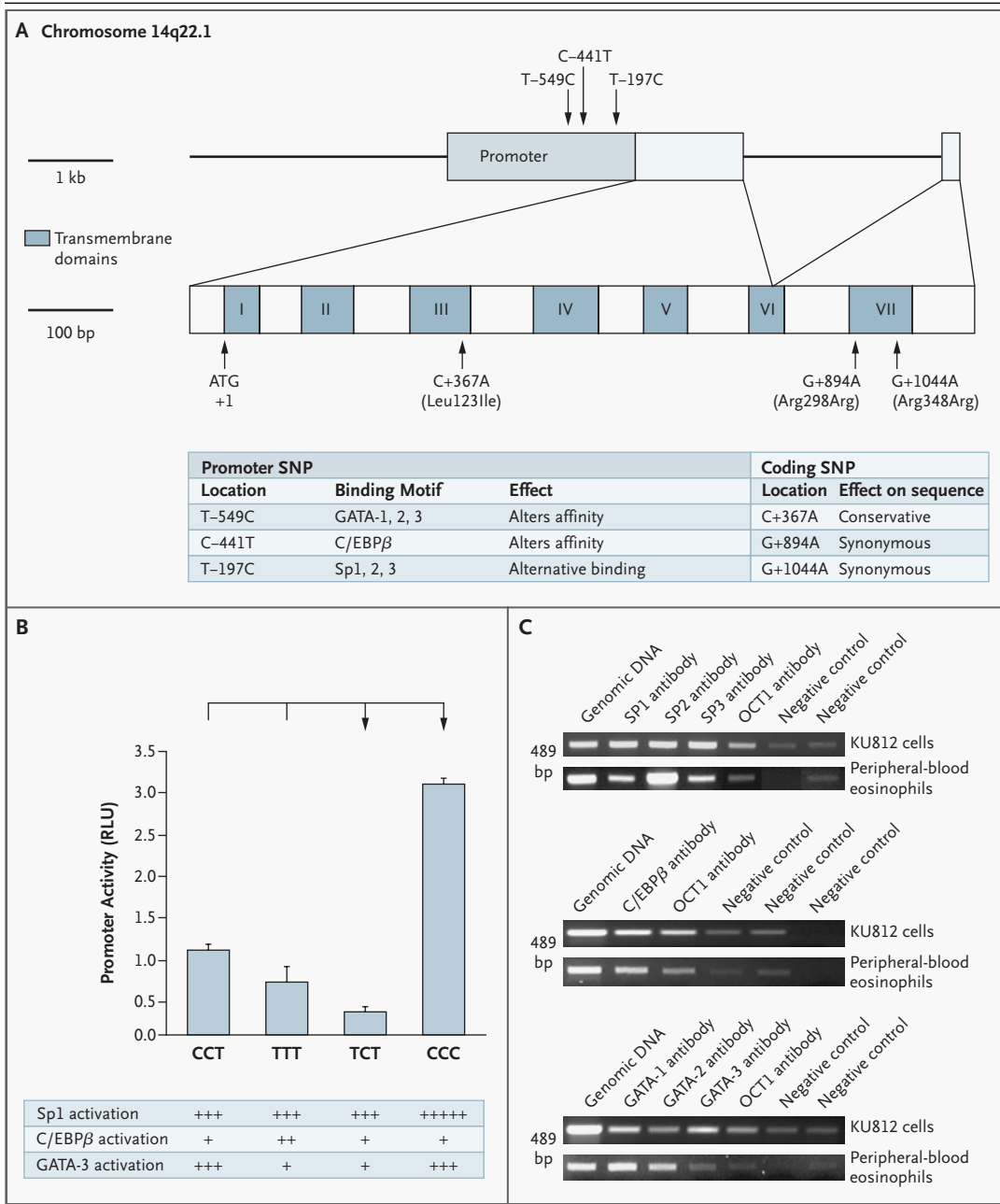
Individual genetic variants were associated with different transcription-factor binding affinities or patterns demonstrated in electrophoretic mobility shift assays (Fig. 2). The region containing the -197

**Figure 1 (facing page).** Human *PTGDR* Gene (Panel A), Activity of the Promoter Variants (Panel B), and Chromatin Immunoprecipitation Assay of the *PTGDR* Promoter Region (Panel C).

Panel A shows the location, nucleotide, and predicted amino acid alterations in *PTGDR* attributed to the variants indicated relative to the ATG start site. Transcription-factor binding motifs affected by the promoter variants are indicated below the sequence along with the effects on the predicted amino acid sequence. Panel B shows the reporter constructs containing 1102 bp of the *PTGDR* promoter region and differing only at the sites that define *PTGDR* haplotypic variants. The promoter activity of haplotype TCT is significantly lower and the promoter activity of haplotype CCC is significantly higher than that of haplotypes CCT and TTT ( $P < 0.05$  by Student-Newman-Keuls test [indicated by arrows]). Values are the means ( $\pm$ SE) of five experiments. The degree of activation is indicated by the plus signs, ranging from minimal activation (+) to extremely high levels of activation (++++). These differences in reporter activity are accounted for by differences in the DNA-binding protein affinity. Panel C shows transcription-factor binding to the *PTGDR* promoter detected by PCR amplification of bound DNA. Increased detection is present after binding of antibodies specific for Sp1, Sp2, Sp3, C/EBP $\beta$ , GATA-1, GATA-2, and GATA-3 to proteins attached to chromatin fragments from prostaglandin D<sub>2</sub> receptors expressing basophilic leukemia cells (KU812) and peripheral-blood eosinophils.

variant bound the transcription factors Sp1, Sp2, and Sp3, contained a consensus Sp1-binding motif, and had substantial reporter activity when Sp1 was provided by an expression construct in a cell type that did not express human Sp-family proteins (Fig. 2A). The electrophoretic mobility supershift assay demonstrated that the alternative sequences bound Sp-family proteins but that the T-containing variant sequence bound an additional DNA-binding protein that did not have Sp1, Sp2, or Sp3 immunoreactivity (Fig. 2A, top, row B).

The region that contains the -441 variant is activated by C/EBP $\beta$ , a transcriptional activator for which there was C/EBP $\beta$  binding to band B of the T-containing variant but not to the C-containing form (Fig. 2B, top, row B). The region containing the -549 variant binds GATA-family transcription factors. It is activated by the asthma-associated transcription factor GATA-3,<sup>25</sup> and the preferential binding of GATA-2 and GATA-3 to the variant (C-containing) form is evident on the electrophoretic mobility supershift assay (Fig. 2C, arrow in top panel and supershift assay in middle panel).

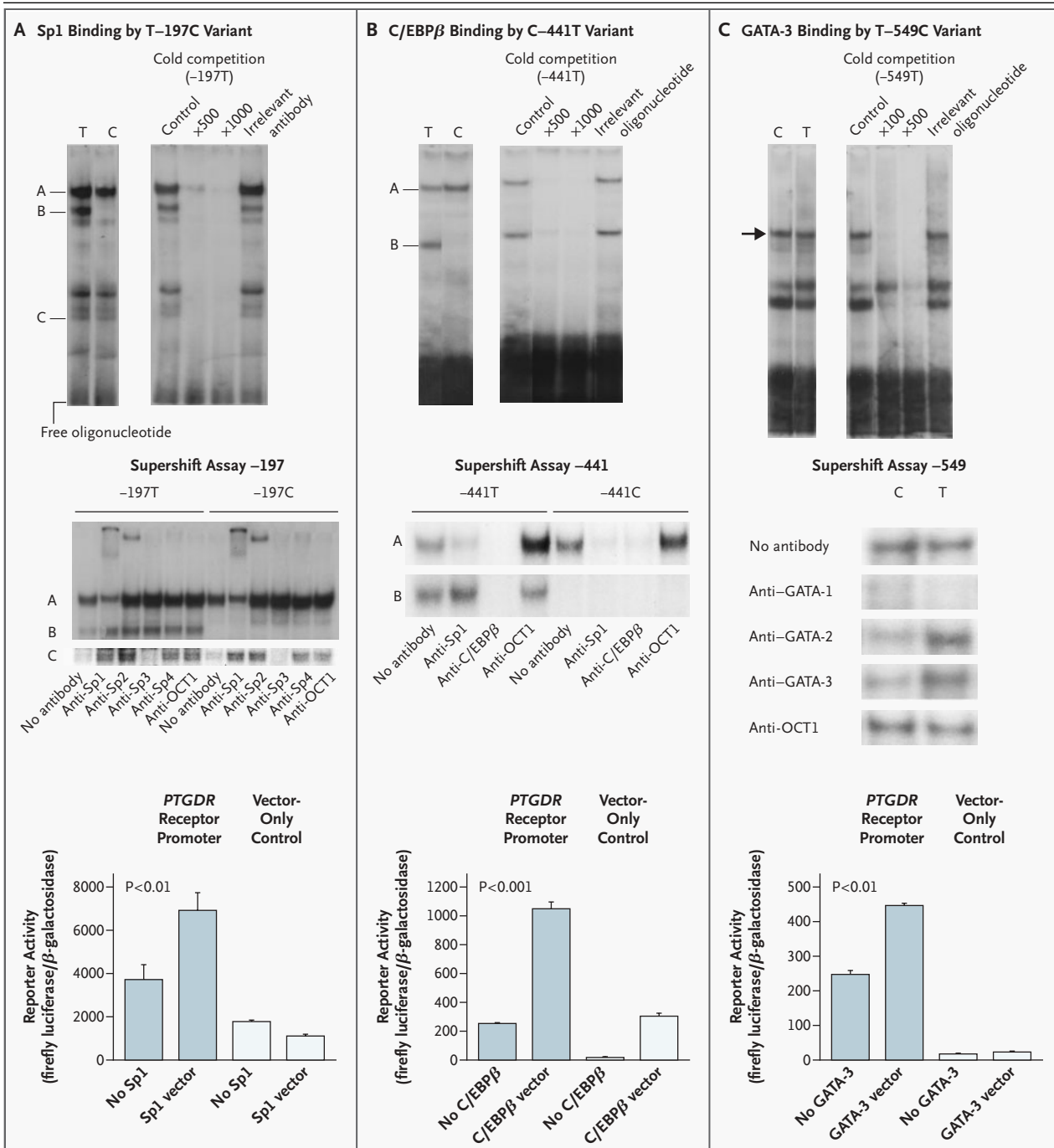


The differences in transcriptional efficiency are accounted for by the associated differences in transcription-factor binding (Fig. 1B).

Assay by chromatin immunoprecipitation showed that Sp1, Sp2, Sp3, C/EBPβ, GATA-1, GATA-2, and GATA-3 bind to the PTGDR promoter of human basophilic leukemia (KU812) cells and peripheral-blood eosinophils (Fig. 1C).

**STUDY POPULATIONS**

The characteristics of our populations are presented in Table 1, and the genotype and allele frequencies of the four PTGDR SNPs analyzed are presented in Table 2. The distribution of genotypes for all the SNPs in both the white and black populations was consistent with the existence of Hardy-Weinberg equilibrium ( $P > 0.05$ ).



**Figure 2. Electrophoretic Mobility Shift Assays Demonstrating the Effects of the Variants on the Binding Profiles of Transcription Factors.**

Panel A shows specific binding of nuclear proteins to the T-197C region of the *PTGDR*. Band B preferentially binds the T form (Panel A, top). Binding of Sp1, Sp2, and Sp3 is demonstrated by supershift assay (Panel A, middle). The reporter activity of the wild-type *PTGDR* is significantly increased by the overexpression of Sp1 in a transactivation assay (Panel A, bottom). Panel B shows specific binding of nuclear proteins to the C-441T region of the *PTGDR*. Band B preferentially binds the T form (Panel B, top). Binding C/EBP $\beta$  is demonstrated by supershift assay (Panel B, middle). The reporter activity of the wild-type *PTGDR* is significantly increased by the overexpression of C/EBP $\beta$  (Panel B, bottom). The T-549C region of *PTGDR* specifically binds nuclear proteins (Panel C, top) that include GATA family members. GATA-2 and GATA-3 preferentially bind the C form (Panel C, middle). The reporter activity of the wild-type *PTGDR* is significantly increased by the overexpression of GATA-3 (Panel C, bottom). OCT-1 denotes octamer transcription factor 1.

**Table 2. Frequencies of PTGDR Sequence Variants among the Patients and Controls in Each Population.**

SNP	Genotype		Allele			
	Controls	Patients	Controls		Patients	
			<i>number of subjects (percent)</i>			
<b>Whites</b>						
T-197C	TT	145 (83)	398 (77)			
	TC	28 (16)	112 (22)			
	CC	2 (1)	8 (2)			
			T	C	T	C
			318 (91)	32 (9)	908 (88)	128 (12)
C-441T	CC	100 (57)*	278 (54)			
	CT	66 (38)	198 (38)			
	TT	9 (5)	42 (8)			
			C	T	C	T
			266 (76)	84 (24)	754 (73)	282 (27)
T-549C	TT	55 (31)†	125 (24)			
	TC	86 (49)	264 (51)			
	CC	34 (19)	129 (25)			
			T	C	T	C
			196 (56)	154 (44)	514 (50)	522 (50)
G+1044A	GG	144 (82)	402 (78)			
	GA	31 (18)	109 (21)			
	AA	0	7 (1)			
			G	A	G	A
			319 (91)	31 (9)	913 (88)	123 (12)
<b>Blacks</b>						
T-197C	TT	40 (89)	61 (76)			
	TC	5 (11)	19 (24)			
	CC	0	0			
			T	C	T	C
			85 (94)	5 (6)	141 (88)	19 (12)
C-441T	CC	22 (49)	34 (42)			
	CT	21 (47)	40 (50)			
	TT	2 (4)	6 (8)			
			C	T	C	T
			65 (72)	25 (28)	108 (68)	52 (32)
T-549C	TT	17 (38)‡	12 (15)			
	TC	18 (40)	49 (61)			
	CC	10 (22)	19 (24)			
			T	C	T	C
			52 (58)	38 (42)	73 (46)	87 (54)
G+1044A	GG	41 (91)	66 (82)			
	GA	4 (9)	14 (18)			
	AA	0	0			
			G	A	G	A
			86 (96)	4 (4)	146 (91)	14 (9)

\* P=0.01 for the comparison with CT and P=0.02 for the comparison with TT, with the use of a multivariate analysis adjusted for age and sex.

† P=0.004 for the comparison with TC and P=0.001 for the comparison with CC, with the use of a multivariate analysis adjusted for age and sex.

‡ P=0.04 for the comparison with both TC and CC, with the use of a multivariate analysis adjusted for age and sex.

**POPULATION STRATIFICATION ANALYSIS**

The analysis of 29 unlinked SNPs suggested no evidence of a significant population substructure within the white case-control population ( $\chi^2_{29}=15.2$ ,  $P=0.98$ ) or the black case-control population ( $\chi^2_{29}=23.5$ ,  $P=0.75$ ).

**GENETIC ASSOCIATION ANALYSES**

The C allele of the T-549C SNP was significantly more common among patients with asthma than among the controls in the white population ( $\chi^2_1=4.27$ ,  $P=0.04$ ) (Table 2). The C allele of the T-549C SNP was also more common among patients with asthma than controls in the black population, although this association did not reach statistical significance ( $\chi^2_1=3.40$ ,  $P=0.06$ ) (Table 2). At the genotypic level, genotypes containing the C allele of the T-549C SNP were more common among patients than controls in both the white population ( $\chi^2_2=4.43$ ,  $P=0.11$ ) and the black population ( $\chi^2_2=8.90$ ,  $P=0.01$ ) (Table 2).

Multivariate analysis of the genotypes adjusted for age and sex confirmed the association of the T-549C SNP with an increased risk of asthma in both the white population (odds ratio for TC as compared with TT, 2.06; 95 percent confidence interval, 1.26 to 3.35;  $P=0.004$ ; and odds ratio for CC as compared with TT, 3.03; 95 percent confidence interval, 1.59 to 5.77;  $P=0.001$ ) and the black population (odds ratio for TC as compared with TT, 3.83; 95 percent confidence interval, 1.07 to 13.75;  $P=0.04$ ; and odds ratio for CC as compared with TT, 6.47; 95 percent confidence interval, 1.07 to 39.15;  $P=0.04$ ). Multivariate analysis also revealed a significant association of genotypes containing one or more copies of the T allele of the C-441T SNP with an increased risk of asthma in the white population after adjustment for age and sex (odds ratio for CT as compared with CC, 1.79; 95 percent confidence interval, 1.14 to 2.79;  $P=0.01$ ; and odds ratio for CC as compared with TT, 3.02; 95 percent confidence interval, 1.24 to 7.32;  $P=0.02$ ). The relationship between asthma and the C-441T SNP was not significant in the black population (odds ratio for CT as compared with CC, 3.23; 95 percent confidence interval, 0.83 to 12.62;  $P=0.09$ ; and odds ratio for CC as compared with TT, 9.04; 95 percent confidence interval, 0.67 to 122.65;  $P=0.10$ ).

White subjects who were carriers of both the C allele of the T-549C SNP and the T allele of the C-441T SNP (26 percent of patients and 18 percent

of controls) had a higher risk of asthma than those who did not carry this combination of alleles (odds ratio, 2.86; 95 percent confidence interval, 1.20 to 6.81;  $P=0.02$ ). We observed similar relationships in the black population; carriers of both the C allele of the T-549C SNP and the T allele of the C-441T SNP (42 percent of patients and 29 percent of controls) had a higher risk of asthma than those who did not carry this combination of alleles (odds ratio, 24.36; 95 percent confidence interval, 1.90 to 312.18;  $P=0.01$ ).

Bivariate analysis with the use of analysis of variance failed to detect a significant association between genotypes at any of the four SNPs and log-transformed values of total IgE in either the white population ( $P>0.28$  for all comparisons) or black population ( $P>0.19$  for all comparisons). Multivariate analysis yielded similar results.

Association analysis according to haplotype demonstrated significant differences in haplotype frequency between patients and controls in both the white population ( $P=0.002$ ) and the black population ( $P=0.01$ ) (Table 3). Similarly, we found significant differences in the diplotype frequency between patients and controls in both the white population ( $P=0.001$ ) and the black population ( $P=0.01$ ) (Table 3).

With respect to haplotype, we found that whites who had at least one copy of the haplotype with a low transcriptional efficiency — TCT — were less likely to have asthma than were whites who had no copies (odds ratio, 0.55; 95 percent confidence interval, 0.38 to 0.80;  $P=0.002$ ). These findings were confirmed in the black population (odds ratio, 0.32; 95 percent confidence interval, 0.12 to 0.89;  $P=0.03$ ). Conversely, whites who had at least one copy of the high-transcriptional-efficiency haplotype CCC were more likely to have asthma than were whites with no copies (odds ratio, 1.53; 95 percent confidence interval, 0.95 to 2.47;  $P=0.08$ ); this difference approached statistical significance. Patients and controls with the diplotype TCT/CCC composed of one low-transcriptional-efficiency haplotype and one high-transcriptional-efficiency haplotype had odds ratios that did not significantly differ from 1.

---

**DISCUSSION**

---

We have found that genetic variants that influence the transcription of *PTGDR* are present in nearly 35 percent of chromosomes in two genetically distinct

U.S. populations and that combinations of individual variants that impair the expression of *PTGDR* are associated with a reduced risk of asthma. Large-scale studies of *PTGDR* variants in population-based samples are now needed to quantify the proportion of asthma risk that is accounted for by these variants in populations with the disease.

A limitation of asthma-susceptibility studies that rely on association alone is that they do not provide information on the mechanism by which the variant leads to the disease.<sup>26</sup> Our findings of association are supported by consistent mechanistic evidence. One of the best-studied mechanisms by which SNPs influence gene expression is that of altering the binding of transcription factors. Consistent with this is our finding that *PTGDR* SNPs that predict asthma occur in regions of its promoter that bind transcription factor *C/EBPβ* and members of the *Sp* and *GATA* families. We established that these transcription factors increase *PTGDR* transcription and that the different SNPs modify their binding; we also identified a combination of variants that decreased transcription.

We based our statistical approach to the genotype–phenotype association on the biologically derived hypothesis that disease association is related to the functional effects of combinations of individual *PTGDR* variants. The observation that combinations of variants had larger functional effects than individual variants suggested that functional combinations might better explain susceptibility to asthma. In support of this hypothesis, we found evidence of an interaction between two of the SNPs in their association with asthma. We therefore chose to examine the disease association of haplotypes that significantly increased or decreased receptor transcription.

That combinations of variants with comparatively large effects were more closely associated with asthma than individual SNPs indicates a strength of our approach: the ability to limit the number of empirical comparisons and prespecify the likely direction of the association. We cannot exclude the possibility that other functional variants are in linkage disequilibrium with the ones that we report, but our results imply that the lower frequency of asthma in subjects who had the TCT haplotype can be explained by the relative inability of these persons to bind the transcription factors that allow the expression of *PTGDR*.

It is well established that the *PTGDR* ligand

**Table 3. *PTGDR* Haplotype and Diplotype Frequencies among Patients and Controls in Each Population.**

Variable	Whites		Blacks	
	Controls	Patients	Controls	Patients
	<i>number of subjects (percent)</i>			
<b>Haplotype*</b>				
CCT	124 (36)	400 (39)	33 (37)	69 (43)
TTT	83 (24)	279 (27)	25 (28)	52 (33)
TCT	111 (32)	229 (22)	27 (30)	21 (13)
CCC	30 (9)	126 (12)	5 (6)	17 (11)
<b>Diplotype†</b>				
CCT CCT	16 (9)	78 (15)	7 (16)	10 (13)
CCT TTT	27 (16)	106 (21)	12 (27)	27 (34)
CCT TCT	48 (28)	93 (18)	4 (9)	12 (15)
CCT CCC	17 (10)	45 (9)	3 (7)	9 (11)
TTT TTT	9 (5)	43 (8)	2 (4)	7 (9)
TTT TCT	34 (20)	60 (12)	8 (18)	6 (8)
TTT CCC	4 (2)	28 (5)	1 (2)	6 (8)
TCT TCT	11 (6)	20 (4)	7 (16)	0
TCT CCC	6 (3)	35 (7)	1 (2)	2 (3)
CCC CCC	1 (1)	8 (2)	0	0

\* All haplotypes with a frequency of more than 1 percent among either patients or controls were included. The order of the SNPs in the haplotype was T-549C, C-441T, and T-197C. The empirical P value by Fisher's exact test (Monte Carlo simulation comparing frequencies between patients and controls) was 0.002 for the white population and 0.01 for the black population.

† The empirical P value by Fisher's exact test (Monte Carlo simulation) was 0.001 for the white population and 0.01 for the black population.

prostaglandin D<sub>2</sub> is the most abundant prostanoid in the asthmatic airway after allergen challenge<sup>27</sup> and that applying prostaglandin D<sub>2</sub> to the airways induces key features of the asthma phenotype.<sup>28,29</sup> Moreover, *PTGDR* is present on mast cells and eosinophils, which generate the effector molecules of the asthmatic diathesis. Our findings are consistent with the notion that persons who are less able to respond to the proallergic effects of prostaglandin D<sub>2</sub> by virtue of impaired transcription of *PTGDR* are less likely to have asthma. The importance of this receptor is suggested by mouse models in which the effects of abrogation of the alternative eicosanoid receptors have demonstrated that deficiency in the prostanoid DP receptor has the greatest effect on the expression of the asthma-like phenotype.<sup>30</sup>

The increasing number of asthma-susceptibility genes that have been identified<sup>26,31,32</sup> suggests

that genetic susceptibility involves more than several genes, each having a larger or smaller effect depending on other genetic and environmental cofactors. The identification of these genes puts us into a position to define their utility for predicting asthma susceptibility. It is likely that each validated variant will contribute, along with environmental, demographic, biochemical, cellular, and developmental factors, to improved risk models that may have useful predictive power. Genetic markers such as the *PTGDR* haplotype that are closely associated with asthma but are not tightly linked to asthma-related phenotypes, such as serum IgE levels, may be useful for accurately classifying patients with asthma who do not express the IgE-related phenotype. The effects of the *PTGDR* haplotype on receptor expression imply that this marker will also be useful for explaining variance in patients' respons-

es to the prostanoid DP receptor antagonists that are now entering clinical trials.

Our findings that *PTGDR* sequence variants are responsible for only part of the genetic susceptibility to asthma are compatible with the known genetic biology of asthma in which a number of genes at distinct chromosomal loci are likely to contribute to disease susceptibility.<sup>3</sup> This study is distinct among genetic association studies of asthma in that we have identified a combination of genetic variants that are associated both with effects on gene expression and protection against asthma.

Supported by grants from the National Heart, Lung, and Blood Institute of the National Institutes of Health and the Ministry of Health, Labor, and Welfare of Japan.

Dr. Palmer reports having served as a consultant to Merck, and Dr. Lilly having received grant support from Eli Lilly.

We are indebted to Omer Kalayci, M.D., for his technical assistance.

#### REFERENCES

1. Asher MI, Keil U, Anderson HR, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J* 1995;8:483-91.
2. Weiss KB, Sullivan SD. The health economics of asthma and rhinitis. I. Assessing the economic impact. *J Allergy Clin Immunol* 2001;107:3-8.
3. Palmer LJ, Cookson WO. Genomic approaches to understanding asthma. *Genome Res* 2000;10:1280-7.
4. Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. *J Biol Chem* 1995;270:18910-6.
5. Matsuoka T, Hirata M, Tanaka H, et al. Prostaglandin D2 as a mediator of allergic asthma. *Science* 2000;287:2013-7.
6. Daniels SE, Bhattacharya S, James A, et al. A genome-wide search for quantitative trait loci underlying asthma. *Nature* 1996;383:247-50.
7. Malerba G, Patuzzo C, Trabetti E, et al. Chromosome 14 linkage analysis and mutation study of 2 serpin genes in allergic asthmatic families. *J Allergy Clin Immunol* 2001;107:654-8.
8. A genome-wide search for asthma susceptibility loci in ethnically diverse populations: the Collaborative Study on the Genetics of Asthma (CSGA). *Nat Genet* 1997;15:389-92.
9. Mansour AH, Bishop DT, Markham AF, Morton NE, Holgate ST, Morrison JF. Suggestive evidence for genetic linkage between IgE phenotypes and chromosome 14q markers. *Am J Respir Crit Care Med* 1999;159:1796-802.
10. Hakonarson H, Bjornsdottir US, Halapi E, et al. A major susceptibility gene for asthma maps to chromosome 14q24. *Am J Hum Genet* 2002;71:483-91.
11. Hall IP. The genetics of asthma: candidate gene approaches: gene-environment interactions. *Clin Exp Allergy* 1998;28 Suppl 1:74-6, 80-1.
12. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am Rev Respir Dis* 1987;136:225-44.
13. Nakamura H, Luster AD, Nakamura T, et al. Variant eotaxin: its effects on the asthma phenotype. *J Allergy Clin Immunol* 2001;108:946-53.
14. Guidelines for the diagnosis and management of asthma. Bethesda, Md.: National Heart, Lung, and Blood Institute, 1997:1-86.
15. Shikanai T, Silverman ES, Morse BW, Lilly CM, Inoue H, Drazen JM. Sequence variants in the *FcεR1α* alpha chain gene. *J Appl Physiol* 2002;93:37-41.
16. Sonna LA, Angel KC, Sharp MA, Knapik JJ, Patton JF, Lilly CM. The prevalence of exercise-induced bronchospasm among US Army recruits and its effects on physical performance. *Chest* 2001;119:1676-84.
17. Jedrzkiewicz S, Nakamura H, Silverman ES, et al. IL-1β induces eotaxin gene transcription in A549 airway epithelial cells through NF-κB. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1058-L1065.
18. Silverman ES, Du J, De Sanctis GT, et al. Egr-1 and Sp1 interact functionally with the 5-lipoxygenase promoter and its naturally occurring mutants. *Am J Respir Cell Mol Biol* 1998;19:316-23.
19. Boyd KE, Wells J, Gutman J, Bartley SM, Farnham PJ. c-Myc target gene specificity is determined by a post-DNA binding mechanism. *Proc Natl Acad Sci U S A* 1998;95:13887-92.
20. Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 1992;48:361-72.
21. Slatkin M, Excoffier L. Testing for linkage disequilibrium in genotypic data using the Expectation-Maximization algorithm. *Heredity* 1996;76:377-83.
22. Mehta CR. The exact analysis of contingency tables in medical research. *Stat Methods Med Res* 1994;3:135-56.
23. McCullagh P, Nelder JA. Generalized linear models. 2nd ed. London: Chapman & Hall, 1989:511.
24. Pritchard JK, Rosenberg NA. Use of unlinked genetic markers to detect population stratification in association studies. *Am J Hum Genet* 1999;65:220-8.
25. Ray A, Cohn L. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *J Clin Invest* 1999;104:985-93.
26. Van Eerdewegh P, Little RD, Dupuis J, et al. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 2002;418:426-30.
27. Wenzel SE, Westcott JY, Smith HR, Larsen GL. Spectrum of prostanoid release after bronchoalveolar allergen challenge in atopic asthmatics and in control groups: an alteration in the ratio of bronchoconstrictive to bronchoprotective mediators. *Am Rev Respir Dis* 1989;139:450-7.
28. Johnston SL, Freezer NJ, Ritter W, O'Toole S, Howarth PH. Prostaglandin D2-induced bronchoconstriction is medi-

ed only in part by the thromboxane prostanoid receptor. *Eur Respir J* 1995;8:411-5.

29. Oguma T, Asano K, Shiomi T, et al. Cyclooxygenase-2 expression during allergic inflammation in guinea-pig lungs. *Am J Respir Crit Care Med* 2002;165:382-6.

30. Kobayashi T, Narumiya S. Function of

prostanoid receptors: studies on knockout mice. *Prostaglandins Other Lipid Mediat* 2002;68-69:557-73.

31. Allen M, Heinzmann A, Noguchi E, et al. Positional cloning of a novel gene influencing asthma from chromosome 2q14. *Nat Genet* 2003;35:258-63.

32. Zhang Y, Leaves NI, Anderson GG, et al. Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma. *Nat Genet* 2003;34:181-6.

*Copyright © 2004 Massachusetts Medical Society.*