

Association of asthma with a functional promoter polymorphism in the *IL16* gene

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Background: IL-16, a multifunctional cytokine with increased expression in the airways of asthmatic subjects, inhibits allergic airway inflammation in animal models. A T→C single nucleotide polymorphism (SNP) at the –295 position in the promoter region of the *IL16* gene has been described. **Objective:** We sought to examine the functional significance of this promoter SNP and its relationship to asthma. **Methods:** We examined the effect of the –295 SNP on promoter activity in cell-line (HBE4-E6/E7) transfection experiments. We investigated the association of the *IL16* –295 genotype with asthma among 341 affected sib-pair white families and 184 unrelated nonasthmatic control subjects. We analyzed the association between the *IL16* genotype and asthma using family-based association test and case-control analyses. **Results:** In *in vitro* transfection experiments the T allele in the –295 position was associated with substantially reduced promoter activity compared with the C allele. In the family study the more common T allele at the –295 position was significantly associated with all asthma phenotypes ($P = .002$ to $P = .015$). In the case-control analysis asthmatic subjects were more likely than unrelated nonasthmatic control subjects to have the –295 TT genotype, but this did not reach statistical significance (odds ratio, 1.36; 95% CI, 0.92–2.02). **Conclusions:** The T allele at the –295 position in the *IL16* promoter region is associated with reduced promoter activity relative to the C allele and with asthma in this white

population. Further investigation is needed to delineate the mechanisms underlying these findings and the relationship of the *IL16* –295 genotype to asthma in other populations. (J Allergy Clin Immunol 2006;117:86–91.)

Key words: Asthma, IL-16, genetic epidemiology, family-based association test, atopy, single nucleotide polymorphism

Asthma is a complex genetic disease that results from environmental and genetic factors. Twin studies provided early evidence of genetic influence on the development of asthma,^{1,2} and genome-wide linkage studies have identified multiple chromosomal loci linked to asthma and related phenotypic traits.³ The identification of specific genetic risk factors for asthma, however, has remained difficult, despite numerous studies of candidate genes targeted because of proximity to sites of linkage, their biologic function, or both.

IL-16 is a multifunctional cytokine initially identified as lymphocyte chemoattractant factor.⁴ The *IL16* gene maps to chromosome 15q26.3⁵ and has 2 different transcription variants encoding 2 distinct isoforms, neuronal *IL16* (isoform 2) and leukocyte *IL16* (isoform 1, Fig 1). Reference to *IL16* in this article denotes leukocyte *IL16*. IL-16 is a 631-amino-acid precursor protein that is cleaved by caspase-3 to form the biologically active C-terminal, which is comprised of 121 amino acids.⁶ In the nondiseased state *IL16* mRNA is present almost exclusively in lymphatic tissue and is constitutively expressed at high levels in T lymphocytes.^{7–9} In the presence of inflammation, IL-16 is synthesized by cells other than T lymphocytes, including the airway epithelial cells of asthmatic subjects.¹⁰

In human subjects *IL16* expression in the airways of asthmatic subjects appears to be increased compared with that seen in healthy control subjects.^{10–12} In one study endobronchial biopsy specimens of atopic asthmatic subjects had significantly higher epithelial IL-16 immunoreactivity than biopsy specimens from atopic nonasthmatic subjects and healthy control subjects ($P < .001$).¹⁰ Antigen challenge leads to a greater increase in bronchoalveolar lavage IL-16 protein concentration in atopic asthmatic subjects than in nonasthmatic subjects.^{11–13} In a murine model of allergic asthma, intraperitoneal administration of IL-16 inhibits antigen-induced airway hyper-responsiveness and decreases the number of eosinophils in bronchoalveolar lavage fluid.¹⁴ Together, these data

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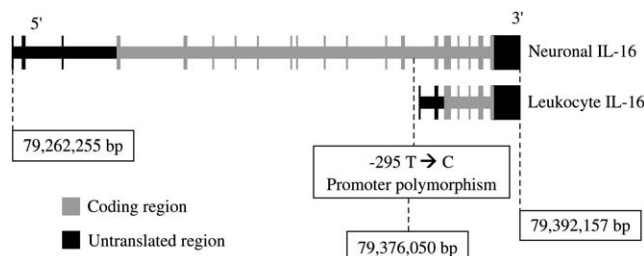


FIG 1. Human *IL16* gene. The *IL16* gene has 2 different transcription variants encoding 2 distinct isoforms, neuronal *IL16* (isoform 2) and leukocyte *IL16* (isoform 1). The $-295\text{ T} \rightarrow \text{C}$ promoter polymorphism is intronic DNA in the coding region of neuronal *IL16*. Promoter regions for *IL16* are not depicted in this figure. Adapted from National Center for Biotechnology Information Entrez Gene: build 35.1; updated January 12, 2005.

Abbreviations used

- ACD: Allergic contact dermatitis
- DTH: Delayed-type hypersensitivity
- FBAT: Family-based association test
- OR: Odds ratio
- SNP: Single nucleotide polymorphism

suggest that the increased levels of IL-16 measured in the airways of asthmatic subjects is a response to, rather than a cause of, increased airway inflammation and that airway epithelial IL-16 has an important immunomodulatory role to inhibit airway inflammation in asthma. Thus *IL16* is an attractive candidate gene for the investigation of genetic risk factors for asthma. We performed cell-culture transfection experiments to examine the functional importance of a recently discovered common single nucleotide polymorphism (SNP) in the promoter region of *IL16*. We also analyzed the association between this SNP and asthma-related phenotypes in a well-characterized sample of white subjects in Southampton, England.

METHODS

Identification of the *IL16* promoter polymorphism

We sequenced the *IL16* promoter region in a sample of 20 coworkers (18 white, 1 Japanese, and 1 Chinese) in Boston, Massachusetts. (The promoter region for leukocyte *IL16* is intronic DNA in the coding region of neuronal *IL16*.) Several SNPs were observed. One of these was a $\text{T} \rightarrow \text{C}$ polymorphism at the -295 position in the promoter (Fig 1), a region with a 90% probability of coding for a GATA-3 binding site, which suggested that this SNP might be of functional importance. In this small convenience sample, the C-allele frequency was 30%. This polymorphism has also been described in Japanese and Thai populations, with a C-allele frequency of 22% and 18%, respectively.¹⁵

Transfection experiments to assess functional significance of -295 SNP

Using human genomic DNA (homozygous -295 T or homozygous -295 C) as a template, we used PCR to amplify the genomic fragment from -1436 to $+31$ of the lymphocyte *IL16* gene. This PCR amplification was performed with the following primers:

5'-ACTTACAGAGCTCCCAACCAGGTGAA-3' and 5'-CACTAGGCTAGCTCCCTGCTGTGTCTTCCTG-3'. *SacI* and *NheI* sites (shown underlined) were added to the 5' ends of the forward and reverse primers, respectively. PCR products for -1436 to $+31$ fragments were separated by means of agarose gel electrophoresis. The PCR products were then cloned into pGL3-Basic vectors with the firefly luciferase reporter. The promoter constructs based on the homozygous -295 TT and homozygous -295 CC DNA templates are called p-295T-luc and p-295C-luc, respectively. All cloning steps were verified by sequencing.

The HBE4-E6/E7 human bronchial epithelial cell line (CRL-2078; American Type Culture Collection, Manassas, Va) was cultured and passed in Keratinocyte-SFM with additives (Invitrogen, Carlsbad, Calif) at 37°C and 5% CO_2 per American Type Culture Collection protocol. Cells cultured to 60% to 70% confluence were transfected with $0.5\text{ }\mu\text{g}$ of either (1) p-295T-luc construct, (2) p-295C-luc construct, or (3) pGL3-Basic vector with no promoter. To normalize transfection rates, cells were cotransfected with 1.0 ng of the Renilla luciferase control vector (pRL-TK; Promega, Madison, Wis). Determination of firefly and Renilla luciferase activities was performed by using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity. We examined relative luciferase activity in unstimulated HBE-E6/E7 cells 24 hours after transfection. The relative luciferase activity of p-295T-luc- and p-295C-luc-transfected cells was analyzed relative to (fold increase) the relative luciferase activity of the cells transfected with pGL3-Basic with no promoter.

Study population

As previously described,¹⁶⁻¹⁹ 341 white affected sib-pair families were recruited from the Southampton, England, area. These families included at least 2 biologic siblings with a current physician diagnosis of asthma and regular use of asthma medications. Both parents were available for all families except one (total = 681 parents). One hundred eighty-four healthy, white, nonasthmatic, adult control subjects with no personal or family history of asthma were recruited from the same Southampton area through blood donor clinics. Ethical approval was obtained from the Southampton, South West Hampshire Joint Ethics Committee and the Institutional Review Board at Boston University Medical Center.

Phenotypic data

Questionnaire data, spirometric data, serum total and specific IgE concentrations, and allergy skin test results were obtained as previously described.¹⁶⁻¹⁹ Asthma diagnosis was defined as a positive response to all of the following questions on the questionnaire: "Have you ever had asthma?"; "Was this confirmed by a doctor?"; and "Have you used any medicine to treat asthma, or any breathing

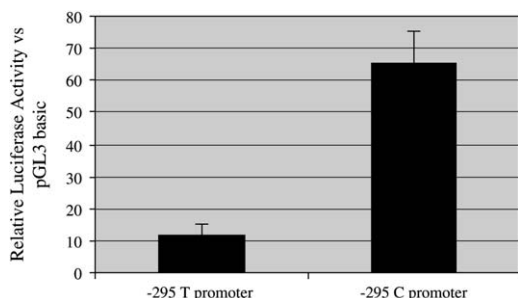


FIG 2. Promoter activity for C and T constructs. The reporter activity of each promoter construct is presented relative to (fold increase) the reported activity for the pGL3-Basic vector. The means and SDs from 5 independent experiments are shown.

problems, at any time in the last 12 months?" Subjects included as nonasthmatic control subjects answered no to all of these questions. Bronchial responsiveness with inhaled methacholine challenge was measured in subjects with a baseline FEV₁ of 70% or more of predicted value. Atopy was defined as a positive skin prick test response (>3 mm) or increased specific IgE level (≥ 0.35 IU/mL) to one or more common allergens. Because self-reported physician-diagnosed asthma might misclassify some subjects, we examined alternative operational definitions of asthma, combining asthma diagnosis with the above measurements.

Genotyping

DNA was extracted from 10 mL of whole blood with a Genomic DNA Maxi-Prep Kit (Qiagen, Crawley, United Kingdom) and frozen at -70°C . A 384-bp region of the *IL16* promoter spanning the -295 SNP was amplified by means of PCR with 24 ng of template DNA with a final concentration in the reaction of 3 mM MgCl₂, 0.2 mM deoxyribonucleoside triphosphates, 0.2 μM of the forward and reverse primers (forward 5'-attgacaagcattttctgagt-3', reverse 5'-cgagacacgctttgattgg-3'), and 0.025 U/ μL of Taq DNA polymerase (Sigma, Poole, United Kingdom). Genotypes for the *IL16* promoter -295 SNP were determined by using an ABI 3700 Automated Sequencer (Applied Biosystems) under standard conditions. SNP detection was carried out with the ABI PRISM SNaPshot Multiplex Kit and the following unlabeled oligonucleotide primer sequence: 5-CCT CCA CAC TCA AAG CCT TTT GTT CCT ATA A-3'.

Data analysis

The association between the *IL16* -295 genotype and various asthma phenotypes was analyzed by using the family-based association test (FBAT) analysis under an additive and biallelic model.²⁰ Because this SNP was selected for study on the basis of a specific biologic hypothesis and not on the basis of genome scan linkage findings, the FBAT analysis was performed presuming no linkage. By using χ^2 analysis, the *IL16* -295 genotypes of the index children were compared with those of the unrelated nonasthmatic control group. χ^2 Goodness-of-fit tests were used to assess whether the observed genotype frequencies differed from those expected under Hardy-Weinberg equilibrium.

RESULTS

Cell transfection study

The human bronchial epithelial cells transfected with the *IL16* promoter construct with a C at the -295 position had substantially greater relative luciferase activity than the cells transfected with the -295 T construct ($65.6 \pm$

10.0 vs 11.6 ± 3.48 , $P < .0001$; Fig 2). These results indicate greater promoter activity for the C construct than the T construct.

Epidemiologic study

The 341 asthmatic children who were the index subjects had a mean age of 13 years and were 57% male (Table I). The 338 second affected siblings had a mean age of 9.9 years and were 54% male. Additional siblings with asthma ($n = 41$) and without asthma ($n = 104$) had a mean age of 9.4 and 12.1 years and were 46.4% and 55.8% male, respectively. The asthmatic and nonasthmatic parents had a mean age of 40 years and were 47% and 51% male, respectively. The nonasthmatic control subjects had a mean age of 42 years and were 47% male. Index asthmatic children, second affected siblings, and asthmatic parents frequently also had diagnosed eczema and hay fever (Table I). In subsequent analyses 24 families were excluded because of apparent genotyping error (nonconformity with Mendelian inheritance) in the *IL16* genotype ($n = 9$), nonpaternity on the basis of multiple genomic markers ($n = 2$), an index asthmatic subject found not to be taking current asthma medications ($n = 1$), and missing genotype data because of failure of PCR amplification ($n = 12$). This allowed for a total of 317 families to be included in the analyses.

The frequency of the *IL16* -295 C allele was 0.167 among the index asthmatic children (Table II) and their affected siblings. The parents had a C-allele frequency of 0.186, and the unrelated nonasthmatic control subjects had a C-allele frequency of 0.21.

The FBAT analysis of the association between the *IL16* -295 allele and asthma among all family members demonstrated a statistically significant association of the *IL16* -295 T allele with all asthma phenotypes (Table III). The strongest associations were for asthma diagnosis ($P = .002$), asthma plus atopy ($P = .004$), and asthma plus increased IgE level ($P = .002$). The multiple asthma phenotypes assessed in the FBAT analysis are correlated outcomes, and therefore the associations observed should not be interpreted as independent associations.

In the case-control analysis 317 unrelated index asthmatic children were more likely than the 162 unrelated nonasthmatic control subjects to have the homozygous -295 TT genotype, although this difference did not reach statistical significance (odds ratio [OR], 1.36; 95% CI, 0.92-2.02). An alternative case-control analysis comparing 181 parents with asthma with the 162 unrelated control subjects revealed an OR of 1.22 (95% CI, 0.79-1.90); however, this analysis has a smaller number of asthmatic subjects and therefore less power than the case-control analysis using the asthmatic probands as cases.

DISCUSSION

These findings indicate that an SNP at the -295 position of the *IL16* promoter is common in the white population in England, with a C-allele frequency of

TABLE I. Characteristics of the study population

	Sibling 1*	Sibling 2†	Other siblings‡		Parents		Control subjects
			Asthmatic	Nonasthmatic	Asthmatic	Nonasthmatic	
No.	341	338	41	104	189	492	184
Age (y)							
Years ± SD	13.0 ± 3.4	9.9 ± 3.1	9.4 ± 2.7	12.1 ± 3.7	40.2	40.7	42.3
Range	6.1-22.6	5-18.1	5.5-18.1	5.3-20.7	28.5-56.2	19.1-60	19-60.3
Sex (%)							
Male	57	54	46	56	47	51	47
Female	43	46	54	44	53	49	52
Eczema§ (%)	58	62	51	34	51	26	0
Hay fever§ (%)	64	47	46	21	70	38	8.7
Positive SPT response (%)	71	63	56	25	79	48	0.5
Increased specific IgE (%)	77	68	66	37	77	48	ND
Total IgE (IU/mL)	5.35 ± 1.7	5.37 ± 1.6	5.27 ± 1.6	4.30 ± 1.6	4.59 ± 1.4	3.80 ± 1.4	ND
FEV ₁ (%) predicted	95 ± 15	96 ± 15	92 ± 13	101 ± 13	94 ± 17	103 ± 13	ND
BHR							
No. of subjects	321	318	39	104	168	487	ND
PC ₂₀ ≤16 mg/mL (%)	66.4	79.3	77.5	38.8	53.6	15.2	
PC ₂₀ <4 mg/mL (%)	46.9	59.9	60.0	19.4	38.1	8.0	

ND, Not done; SPT, skin prick test; BHR, bronchial hyperresponsiveness; PC₂₀, concentration of methacholine needed to provoke a 20% or greater decrease in FEV₁.

*Sibling 1 is index asthmatic child.

†Sibling 2 is second affected sibling.

‡Excluding sibling 1 and sibling 2.

§Positive history on the basis of questionnaire.

||Total IgE: transformed with natural logarithm.

TABLE II. Genotype frequencies of the -295 T/C polymorphism of the *IL16* gene

	Sibling 1* (n = 317)	Parents (n = 648)	Control subjects (n = 162)
TT (%)	216 (68.1)	423 (65.3)	99 (61.1)
TC (%)	96 (30.3)	209 (32.2)	58 (35.8)
CC (%)	5 (1.6)	16 (2.5)	5 (3.1)
f [†] (T)	0.833	0.814	0.790
f [†] (C)	0.167	0.186	0.210

All groups were found not to differ significantly from Hardy-Weinberg equilibrium.

*Sibling 1 is index subject.

†Frequency of allele.

approximately 0.20 and a T-allele frequency of approximately 0.80. Our *in vitro* findings demonstrate functional significance of this promoter-region SNP in transfection experiments using cultured human bronchial epithelial cells. This functional significance might relate to the location of the -295 SNP at a GATA-3 binding site. There are 120 SNPs in the leukocyte *IL16* gene listed on the National Center for Biotechnology Information Web site, of which 22 SNPs have been validated. Of the validated SNPs, 2 are coding synonymous, 9 are untranslated, and 11 are intronic. The -295 SNP is the only validated SNP in the putative promoter region of the leukocyte *IL16* gene.

Analysis of asthmatic children and their families with the FBAT revealed that the T allele was associated with

TABLE III. FBAT analysis of asthma-related phenotypes and *IL16* -295 genotype among affected families with affected siblings

Phenotype	Informative families	P value
Asthma diagnosis	170	.002
Asthma and atopy	172	.004
Asthma and increased total IgE level*	171	.002
Asthma and PC ₂₀ ≤16 mg/mL	169	.015
Asthma and PC ₂₀ < 4 mg/mL	168	.006

FBAT analysis was performed with additive model and biallelic test.

PC₂₀, Concentration of methacholine needed to provoke a 20% or greater decrease in FEV₁.

*Increased serum total IgE concentration defined as a value greater than 1 SD greater than the age group-specific median.

asthma within these families. An analysis comparing index asthmatic children with unrelated nonasthmatic control subjects suggested that the TT genotype was more common among the asthmatic subjects than among the control subjects (OR, 1.36; 95% CI, 0.92-2.02), although this difference did not reach statistical significance. To date, the genetic epidemiologic investigation of asthma has not revealed genetic mutations or polymorphisms that confer large relative risks of asthma. Rather, it appears increasingly likely that there are numerous genetic risk factors, each conferring a small relative risk. Future case-control studies should include larger numbers of subjects than ours to have adequate power to detect odds ratios in the range of 1.25 to 1.5.

Although the role of IL-16 in the pathogenesis of asthma and allergy remains uncertain, growing evidence indicates that this cytokine might be important in these disorders. Increased levels of IL-16 have been demonstrated in human airways of asthmatic subjects compared with levels in atopic nonasthmatic subjects and healthy control subjects.¹⁰⁻¹³ In an animal model of asthma, administration of exogenous IL-16 inhibited antigen-induced airway hyperresponsiveness and decreased airway eosinophils.¹⁴ Yoshimoto et al²¹ investigated the involvement of IL-16 in delayed-type hypersensitivity (DTH) reactions induced by injecting methylated bovine serum albumin into the footpads of mice. The DTH reactions were associated with IL-16 expression in epithelial cells and infiltrating cells that were not present in the footpads injected with diluent alone. Furthermore, treatment of sensitized mice with neutralizing mAb against IL-16 significantly decreased the footpad swelling induced by antigen challenge.²¹ T_H2 cytokines play a fundamental role in the pathogenesis of the airway inflammation in asthma,^{22,23} whereas DTH responses are driven primarily by T_H1 cytokines.^{24,25} These studies suggest that IL-16 might inhibit inflammation in T_H2-mediated hypersensitivity and augment inflammation in T_H1-mediated hypersensitivity.

Several recent reports have suggested that the *IL16* -295 SNP genotype might be a risk factor for inflammatory disorders. In 2002, Glas et al²⁶ reported an association between the *IL16* -295 SNP and Crohn's disease. Subjects with Crohn's disease had significantly higher T-allele and TT-genotype frequencies compared with those seen in control subjects ($P < .01$). An association with the C allele or T allele was not seen in the subjects with ulcerative colitis. In 2003, Reich et al²⁷ described an association of the *IL16* -295 SNP with allergic contact dermatitis (ACD), a DTH response believed to be driven primarily by T_H1 cytokines. In that study the -295 CC genotype was more common in subjects with polysensitized ACD than in a healthy control group without ACD (OR, 7.68; 95% CI, 1.59-48.12). Assuming that the expression of IL-16 or levels of functional IL-16 vary according to *IL16* -295 genotype, it is logical that asthma and ACD would be associated with discordant alleles at the -295 position (ie, TT genotype is associated with asthma, a disease driven by T_H2 cytokines, and CC genotype is associated with ACD, a disease driven by T_H1 cytokines). The recent observation that IL-16 selectively attracts and activates T_H1 cells further implies a possible discordance between diseases dependent on T_H1 versus T_H2 inflammatory processes.²⁸ Recently, Akesson et al²⁹ reported no association between the *IL16* -295 promoter polymorphism and asthma in a case-control study in an Australian white population; however, case-control studies are subject to population stratification, and there was no assessment of possible population admixture in the study. Our family-based association study is not subject to confounding by population stratification. Differences in environmental exposures between populations and different phenotype definitions might also have

contributed to the conflicting findings of our study and that of Akesson et al.

Our cell-transfection experiments indicate that the -295 C allele is associated with increased promoter activity compared with the T allele. This finding suggests that persons with 1 or 2 C alleles might produce more IL-16 than persons who are homozygous for the T allele. The hypothesis, discussed above, that IL-16 inhibits T_H2-mediated hypersensitivity would therefore suggest that the C allele might be protective against asthma relative to the T allele. This is exactly the pattern of association observed in our epidemiologic study.

Although the association of the *IL16* -295 T allele with asthma was statistically significant in family-based analyses, the strength of the association is modest, and the suggested association of the TT genotype with asthma in the case-control analysis (OR, 1.36) was not statistically significant (95% CI, 0.92-2.02). In light of the undoubted complexity of the multiple genetic and environmental influences contributing to the development of asthma, it is to be expected that any single genetic polymorphism contributing to asthma might have only a modest effect. Prior genome-wide linkage studies have not demonstrated linkage to this locus on chromosome 15q. The absence of linkage might in part be explained by its modest effect size and by the complexity of this disease, which likely requires variability in multiple genes, with both gene-by-gene and gene-by-environment interactions.

The finding of an association between the *IL16* -295 SNP and 3 distinct diseases mediated by inflammation (Crohn's disease, ACD, and asthma) suggests that this SNP might be functionally important with respect to inflammatory pathways. Although the previously reported studies of Crohn's disease²⁶ and ACD²⁷ are case-control studies susceptible to potential confounding by population stratification, our findings for asthma in the FBAT analysis are not subject to this limitation. Although our *in vitro* data suggest functional significance of the -295 SNP, it remains possible that the -295 *IL16* genotype is not itself functionally related to asthma but is instead in linkage disequilibrium with another nearby genetic polymorphism that is functionally related to asthma.

In summary, this study demonstrates that the T allele at the -295 position in the *IL16* gene promoter region is associated with reduced promoter activity relative to the less common C allele on transfection into a bronchial epithelium cell line. Our study also demonstrates an association of the TT genotype and T allele at the -295 position in the *IL16* promoter region with asthma in a white population. Further investigation is needed to delineate the mechanisms underlying these findings and the relationship of the *IL16* -295 genotype to asthma in other populations.

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