T-Bet Polymorphisms Are Associated with Asthma and Airway Hyperresponsiveness

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Rationale: T-bet (TBX21 or T-box 21) is a critical regulator of T-helper 1 lineage commitment and IFN- γ production. Knockout mice lacking T-bet develop airway hyperresponsiveness (AHR) to methacholine, peribronchial eosinophilic and lymphocytic inflammation, and increased type III collagen deposition below the bronchial epithelium basement membrane, reminiscent of both acute and chronic asthma histopathology. Little is known regarding the role of genetic variation surrounding T-bet in the development of human AHR.

Objectives: To assess the relationship between T-bet polymorphisms and asthma-related phenotypes using family-based association.

Methods: Single nucleotide polymorphism discovery was performed by resequencing the T-bet genomic locus in 30 individuals (including 22 patients with asthma). Sixteen variants were genotyped in 580 nuclear families ascertained through offspring with asthma from the Childhood Asthma Management Program clinical trial. Haplotype patterns were determined from this genotype data. Family-based tests of association were performed with asthma, AHR, lung function, total serum immunoglobulin E, and blood eosinophil levels.

Main Results: We identified 24 variants. Evidence of association was observed between c.-7947 and asthma in white families using both additive ($p = 0.02$) or dominant models ($p = 0.006$). c.-7947 and three other variants were also associated with AHR (log-methacholine PC₂₀, $p = 0.02$ – 0.04). Haplotype analysis suggested that an AHR locus is in linkage disequilibrium with variants in the 3'UTR. Evidence of association of AHR with c.-7947, but not with other 3'UTR SNPs, was replicated in an independent cohort of adult males with AHR. **Conclusions:** These data suggest that T-bet variation contributes to airway responsiveness in asthma.

Keywords: immunoglobulin E; single nucleotide polymorphism; T-box; TBX21

T-bet (TBX21, Online Mendelian Inheritance of Man database no. 604895) is a nuclear transcription factor belonging to the T-box gene family of DNA-binding proteins, and is a potent inducer of IFN- γ , critical for T-helper type 1/T-helper type 2

(Th1/Th2) differentiation of CD4⁺ lymphocytes (1, 2). In CD4⁺ lymphocytes, T-bet expression is restricted to Th1 populations, and Szabo and colleagues demonstrated that transduction of T-bet in Th2-committed cells redirects these cells toward a Th1 phenotype (1, 2). Gene knockout mice lacking T-bet spontaneously develop histologic and physiologic phenotypes reminiscent of asthma, including peribronchial and perivascular eosinophilic and lymphocytic inflammation, airway hyperresponsiveness (AHR), and increased type III collagen deposition below the bronchial epithelium basement membrane (3). In humans, T-bet expression is markedly decreased in peribronchial CD4⁺ lymphocytes in patients with asthma than in samples from normal control subjects (3). T-bet is also expressed in antigen-presenting cells, including dendritic cells (4), and T-bet is essential for optimal production of IFN- γ by dendritic cells and subsequent antigen-specific Th1 activation *in vivo* (5), suggesting that altered T-bet expression in nonlymphocyte cell types also impacts asthma pathobiology.

Given these data, it is possible that genetic variation at the T-bet locus confers susceptibility to asthma, AHR, and atopy. Two reports evaluating T-bet single nucleotide polymorphisms (SNPs) in asthma populations from Korea and Finland failed to demonstrate evidence of a relationship with either a diagnosis of asthma or total serum IgE levels; however, both studies reported they were statistically underpowered to detect genetic associations of modest effect (odds ratios, ~ 2 – 3) (6, 7). A third study in a Japanese population demonstrated associations of one T-bet variant with aspirin-induced asthma, but not with other forms of asthma (8). None of these studies examined relationships between T-bet variants and airway physiology. Presented here is our characterization of T-bet sequence variation and our evaluation of T-bet variants in a family-based association study of childhood asthma and in a second cohort of adult men responsive to methacholine. Our results suggest that T-bet variation affects the AHR phenotype. Preliminary results of our findings have previously been reported in abstract form (9).

METHODS

Populations

The Childhood Asthma Management Program (CAMP) is a multicentered North American clinical trial designed to investigate the long-term effects of inhaled antiinflammatory medications in children with mild to moderate asthma (10, 11). A total of 968 of the 1,041 children enrolled in CAMP and 1,518 of their parents contributed DNA samples. A total of 580 complete nuclear families are included in the analysis presented here. See online supplement for additional details for inclusion criteria and phenotyping protocols.

The Normative Aging Study (NAS) is a longitudinal study of aging established by the Veterans Administration in 1961 (12). The initial

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cohort consisted of 2,280 men from the greater Boston area ages 21 to 80 yr at the time of entry into the study between 1961 and 1969. Since entry, volunteers have reported for periodic examinations that include spirometric tests and modified methacholine challenge test. Methacholine responders were identified by a fall in FEV₁ of 20% or more from baseline during the challenge test. Nonresponders demonstrated a less than 20% fall in FEV₁. DNA for 200 responders and 436 nonresponders was available for genotyping. *See* online supplement for additional details.

Human Subjects

The Institutional Review Board of the Brigham and Women's Hospital and those of the other CAMP study centers approved these studies. Genetic studies in the NAS were approved by the Partners Healthcare Human Research Committee and the Institutional Review Board of the Veterans Administration Hospitals using anonymized datasets.

Polymorphism Discovery and Genotyping

The T-bet locus was resequenced in 30 white subjects (22 with asthma, 8 without) with dye-terminator dideoxy sequencing chemistry (PE Biosystems, Foster City, CA). SNPs were genotyped using unlabeled mini-sequencing reactions and mass spectrometry analysis as implemented in the SEQUENOM platform (Sequenom, San Diego, CA), and by TaqMAN assays (PE Biosystems) (13). We developed reliable assays for 12 of 24 identified T-bet variants and for four additional variants from the public database dbSNP and from previous reports: g.-19698C > A, His33Gln, Pro485Pro, and g.56983A > G. *See* online supplement for additional details.

Statistical Analysis

Hardy-Weinberg equilibrium and pairwise linkage disequilibrium (LD) were assessed using parental (CAMP) and control (NAS) genotype data (14–16). Haplotype block structure analysis as defined by Gabriel ($D' > 0.9$; minimum allele frequency, 5%) was performed with Haploview (17, 18).

FBAT version 1.5.3 (19, 20) was used for family-based association analysis with asthma in CAMP. Quantitative trait analysis was performed with principal components analysis as implemented in PBAT (FBAT-PC) (21, 22). Evidence for haplotype association was assessed with the likelihood-ratio score test implemented in TRANSMIT (23) (asthma) and FBAT (quantitative traits) (24). *See* online supplement for additional details.

NAS data were analyzed with SAS Genetics (SAS Institute, Cary, NC) and used generalized linear models for analysis of methacholine responsiveness (log-transformed dose–response slope). Models were adjusted for age, pack-years of cigarette smoking, and current smoking status. *See* online supplement for additional details.

RESULTS

SNP Discovery

Resequencing of the T-bet locus identified 24 SNPs, including six variants in the 22 people with asthma and 18 variants in the people without asthma (*see* Figure E1 in the online supplement). Only one nonsynonymous variant was identified (Ile339Val), observed once in an individual without asthma. Virtually none of the SNPs identified reside in highly conserved regions: none were identified in the highly conserved T-box domain, and the only SNP identified in a conserved region of the transcript, Gly130Gly, was synonymous. One upstream variant, c.-7947A > G, did localize to a region of modest phylogenetic conservation, although the minor allele at this locus is present in rodent sequence.

SNP Genotyping, Linkage Disequilibrium, and Haplotype Block Structure in CAMP

Baseline characteristics of the 632 CAMP probands are presented in Table 1. Sixteen T-bet SNPs were genotyped. All genotyped polymorphisms were in Hardy-Weinberg equilibrium. Table 2 shows the minor allele frequency for each locus in the parents of the CAMP children, along with available allele frequency data from previously published reports. Pairwise LD (expressed as r^2) for the 16 SNPs typed in the white CAMP parents is presented in Figure 1a. SNPs at the 5' end (including c.-1993, c.-1514, Gly130, IVS1+238, and IVS1+2354) and others at the 3' end of the gene (including Pro485, c.2122, g.21126, and g.56983) demonstrate modest to strong degrees of LD with one another, but substantially less LD with those at opposite ends of the locus. Some overlap is noted, however, with several 5' SNPs demonstrating strong LD with the more downstream IVS5-22 and c.2314. Figure 1b displays the imputed haplotype block structure across the T-bet locus. Two blocks are identified, with restricted haplotype diversity within each block. Of note, multiallelic D' between blocks was high (~ 0.95), as evidenced by restricted diversity of the combination haplotypes formed between blocks (i.e., meta-haplotypes). g.-19698 and c.-7947 are not in strong LD with more proximally situated variants and do not form part of the haplotype block structure at the T-bet locus. The LD patterns in the African-American and Hispanic

TABLE 1. BASELINE CHARACTERISTICS OF CAMP PROBANDS GENOTYPED FOR T-BET VARIANTS

Variable	Median (interquartile range) or Count (%)		
	White (n = 507)	African American (n = 70)	Hispanic (n = 55)
Age, yr	8.86 (7.2–10.4)	9.2 (7.0–10.8)	9.3 (7.5–10.9)
Sex			
Male	313 (61.7%)	45 (64.3%)	39 (70.9%)
Female	194 (38.3%)	25 (35.7%)	16 (29.1%)
Treatment arm			
Budesonide	139 (27.4%)	19 (27%)	18 (32.7%)
Nedocromil	152 (30.0%)	19 (27%)	16 (29.1%)
Placebo	216 (42.6%)	32 (45.7%)	21 (38.2%)
Age of asthma symptom onset, yr	2.3 (1.0–4.0)	2.25 (1.0–5.0)	3.0 (1.1–5.0)
Methacholine PC ₂₀ , mg/ml	0.74 (0.43–1.52)	0.78 (0.51–1.98)	0.91 (0.53–1.75)
Postbronchodilator FEV ₁ , L	1.72 (1.41–2.21)	1.63 (1.36–1.91)	1.78 (1.47–2.25)
Postbronchodilator FEV ₁ , % pred	100% (94–109%)	102% (91.0–108%)	102% (92–111%)
Postbronchodilator FEV ₁ /FVC ratio	0.86 (0.83–0.92)	0.86 (0.81–0.91)	0.85 (0.79–0.89)
Total serum IgE levels, ng/ml	938 (338–1,882)	511 (288–1,169)	717 (294–1,716)
Blood eosinophil levels, cells/mm ³	230 (1.105–458)	400 (198–620)	296 (100–475)
At least one positive skin-prick test, ≥ 3 mm	438 (86.4%)	63 (90.0%)	54 (98.2%)

Definition of abbreviation: CAMP = Childhood Asthma Management Program.

TABLE 2. T-BET ALLELE FREQUENCIES IN CAMP PARENTS

SNP	dbSNP	Alleles*	CAMP			Chung and colleagues (6)			Ylikoski and colleagues (7)	
			White (n = 944)	Hispanic (n = 92)	African American (n = 129)	Korean (n = 24 or 990)	African American (n = 50)	White (n = 50)	High IgE (n = 277)	Asthma (n = 199)
g.-19698	rs2013383	G > T	0.424	0.467	0.252					
c.-7947	rs9910408	A > G	0.432	0.457	0.287					
c.-1993	rs4794067	T > C	0.269	0.360	0.190				0.265	0.278
c.-1514	—	T > C	0.183	0.173	0.125	0.063	0.110	0.140	0.191	0.184
His33Gln	rs2240017	C > G	0.027	0.071	0.004	0.118	0.020	0.030		
Gly130Gly	rs2074190	A > G	0.268	0.358	0.300	0.091	0.350	0.250	0.270	0.282
IVS1+238	—	G > T	0.182	0.149	0.072					
IVS1+2354	—	G > A	0.185	0.136	0.113					
IVS1+7240	rs2158079	T > C	0.113	0.190	0.027					
IVS4-4	—	C > G	0.058	0.047	0.013				0.018	0.025
IVS5-22	rs11650354	C > T	0.186	0.125	0.106	0.054	0.100	0.140	0.188	0.186
Pro485Pro	—	G > A	0.027	0.124	0.055	0.043	0.061	0.050	0.058	0.075
c.2122	—	G > A	0.071	0.154	0.058				0.067	0.083
c.2314	rs7502875	A > C	0.236	0.159	0.231				0.180	0.175
g.21126	—	C > T	0.076	0.163	0.091					
g.56983	rs2325717	T > C	0.067	0.172	0.031					

Definition of abbreviations: CAMP = Childhood Asthma Management Program; Gln = glutamine; Gly = glycine; His = histidine; IVS = intervening sequence (intron); Pro = praline; SNP = single nucleotide polymorphism.

* Allele frequencies presented are those of second allele listed. SNPs are named relative to A of initiation codon in refseq NM_013351, relative to chromosome 17 reference sequence (May 2004 freeze) at genome.ucsc.edu.

samples are very similar to those observed in the white samples, although LD is stronger at the 5' end of the T-bet locus in whites (see online supplement Figures E2a and E2b).

SNP Association Analysis

Presented in Table 3 are the results of the single-SNP, family-based analysis of association with the asthma phenotype and with AHR among whites. Only 1 of 16 SNPs tested demonstrated evidence of association with asthma: c.-7947G was undertransmitted to affected offspring. Under the additive model, this distortion has a p value, uncorrected for multiple comparisons, of 0.02. Stronger evidence for association was found at this locus

with a dominant model ($p = 0.006$), although such modeling was performed *post hoc*. No SNP mapping to the transcript itself demonstrated significant association with asthma, although the nonsynonymous His33Gln polymorphism demonstrated the most skewed transmission distortion for all SNPs tested, with a transmitted:untransmitted ratio of approximately 3:2 for the Gln allele ($p = 0.28$). Similar patterns of transmission distortion were observed when analysis was restricted to those probands with at least one positive skin-prick test. No association with asthma was found in either the African-American or Hispanic families, although sample size in these two cohorts was insufficient to detect associations of modest effect (data not shown).

Figure 1. T-bet linkage disequilibrium and haplotype block structure in white Childhood Asthma Management Program (CAMP) parents. (a) Pairwise linkage disequilibrium plots (r^2) for 16 variants genotyped in white CAMP parents. Arrow denotes relative position of T-bet gene (not to scale). (b) T-bet haplotype block structure in the white CAMP parents was estimated with Haploview using the Gabriel block definition using single nucleotide polymorphisms (SNPs) with minor allele frequencies of at least 0.05 (18). SNPs His33Gln and Pro485Pro have minor allele frequencies of less than 0.05 and are therefore excluded from this analysis. Haplotypes approximating 5% are displayed (frequencies adjacent to blocks). Thin and thick lines connecting blocks denote between-block combinations observed at least at 1% or 10%, respectively. Arrows denote one of several SNP subsets that tag the haplotypes within each block.

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Figure 2. Relationship between T-bet SNPs g.-19698 (a) and c.-7947 (b) and methacholine dose-response slope among 199 methacholine responders in the Normative Aging Study. Dose-response slope is adjusted for age, pack-years of smoking, and current smoking status. Results remained significant after adjustment for baseline FEV₁. Minor allele frequencies in this cohort are as follows: g.-19698 = 0.370; c.-7947 = 0.382; His33Gln = 0.041; IVS1+7240 = 0.208; c.2122 = 0.070; c.2314 = 0.424.

Repeated measures of postbronchodilator FEV₁ (10–12 measurements/participant), methacholine PC₂₀ (2–5 measurements), total serum IgE levels, and blood eosinophil levels (2 measurements each) were obtained over the 4.5 yr of observation during the CAMP trial. In whites, no evidence for association was found with FEV₁, IgE, or eosinophils. However, several SNPs across the T-bet locus were associated with AHR in CAMP, including c.-7947, one variant localized to the T-bet transcript (c.2122), and two 3' genomic variants (g.21126 and g.56983). Of these associations, those with c.2122 and g.21126 met conservative criteria for significance (rank-order Bonferroni corrected p values of 0.04 and 0.02, respectively). Because of

the small number of informative families in other ethnic groups, quantitative analysis was restricted to the white cohort.

Haplotype Association Analysis

We performed haplotype association analysis in the white families using a subset of six polymorphisms that tag all observed haplotypes within each block with a frequency of 5% or greater (*arrowheads* in Figure 1b). To minimize the number of tests performed, we first screened for evidence of global haplotype association using omnibus test statistics. Only those haplotype groups significantly associated by this screening technique were then examined for specific haplotype associations. No haplotype

TABLE 3. ASTHMA ASSOCIATION AND QUANTITATIVE TRAIT ANALYSIS FOR T-BET SINGLE NUCLEOTIDE POLYMORPHISMS IN WHITE CAMP FAMILIES

SNP	Allele	Asthma Association Analysis			Quantitative Trait Analysis†		
		FBAT p Value			FBAT-PC p Value		
		T:U*	Additive Model	Dominant Model	logPC ₂₀	FEV ₁ (% pred)	logIgE
g.-19698	T	271:238	0.14	0.14	0.72	0.62	0.12
c.-7947	G	230:281	0.02	0.006	0.03	0.10	0.16
c.-1993	C	149:167	0.31	0.43	0.28	0.16	0.61
c.-1514	C	129:126	0.85	0.80	0.30	0.49	0.78
His33Gln	G	31:23	0.28	0.30	0.33	0.35	0.12
Gly130Gly	G	184:177	0.71	0.82	0.16	0.54	0.58
IVS1+238	T	116:118	0.90	0.51	0.39	0.31	0.70
IVS1+2354	A	126:127	0.95	0.75	0.22	0.17	0.95
IVS1+7240	C	107:86	0.13	0.09	0.29	0.34	0.04
IVS4-4	G	51:46	0.61	0.43	0.18	0.19	0.24
IVS5-22	T	138:139	0.95	0.92	0.24	0.79	0.74
Pro485Pro	A	21:20	0.88	1.00	0.07	0.27	0.44
c.2122	A	60:69	0.43	0.52	0.02‡	0.08	0.12
c.2314	C	143:160	0.33	0.28	0.07	0.21	0.67
g.21126	T	60:71	0.34	0.52	0.02‡	0.13	0.17
g.56983	C	53:60	0.51	0.62	0.04	0.09	0.25

Definition of abbreviations: CAMP = Childhood Asthma Management Program; SNP = single nucleotide polymorphism.

* T:U denotes ratio of transmitted to untransmitted allele counts. T:U counts were derived using Haploview (17). p values are not corrected for multiple comparisons.

† An additive genetic model was assumed for quantitative trait analysis.

‡ The c.2122- and g.21126-logPC₂₀ associations remained significant after rank-ordered Bonferroni correction based on power, as implemented in PBAT (p-adjusted 0.04 and 0.02, respectively) (21, 22).

associations were observed with asthma, FEV₁, IgE, or eosinophils (data not shown). However, significant haplotype associations with AHR were observed (Table 4). The screening test demonstrated association of the meta-haplotypes with AHR (multiallelic $p = 0.02$), and screening of individual blocks suggested that the principal associations with AHR localized to block 2 (multiallelic $p = 0.04$), not block 1 ($p = 0.59$). Within block 2, associations were restricted to haplotype H7, uniquely tagged by c.2122. It is therefore likely that the principal associations with AHR in this block are with c.2122 or other variants in tight LD on the H7 haplotype background.

Replication of T-bet Association with AHR

We attempted to replicate evidence for an association between T-bet variants and AHR in a second population: 200 methacholine-hyperresponsive (responders) and 436 nonresponsive men participating in the NAS (12). Baseline characteristics are presented in Table 5. Methacholine responders were older, were more likely to have previously smoked, had lower baseline lung function, had higher total serum IgE levels and blood eosinophil levels, and were more likely to have a positive skin test to one of four common aeroallergens. Six variants were genotyped: g.-19698, c.-7947, His33Gln, IVS1+7240, c.2122, and c.2314. All were in Hardy-Weinberg equilibrium. None of these SNPs differentiated responders from nonresponders (data not shown). Among the responsive subjects, a strong association was found between methacholine responsiveness and c.-7947 (Figure 2). The other five variants were not associated with AHR in NAS, although trends were observed for g.-19698 ($p = 0.06$ for common vs. rare homozygote genotypes). No associations with total serum IgE or blood eosinophil levels were observed in this cohort.

DISCUSSION

T-bet serves as a regulator of Th1 development both by inducing IFN- γ production and by inhibiting Th2 cytokines interleukin (IL)-4 and IL-5 (reviewed by Murphy and Glimcher [25]). The T-bet locus maps to chromosome 17q21, a region occasionally

linked with both asthma and atopy-related phenotypes (26, 27). We provide data demonstrating a potential association between T-bet variants and AHR. One variant, c.-7947, was found to be weakly associated with asthma susceptibility, and four variants dispersed across the T-bet locus (c.-7947, c.2122, c.21126, and c.56983) were significantly associated with AHR in children with asthma. We have also provided some evidence that the relationship between c.-7947 and airway responsiveness is reproducible in a second cohort of older men with AHR, although confirmation of this and other associations in additional populations is warranted before firm conclusions regarding the generalizability and true impact of these findings can be drawn. No associations were found with FEV₁, serum IgE levels, or blood eosinophil levels in either cohort.

This study complements earlier work in which we demonstrated a significant pharmacogenetic effect of the His33Gln variant in the long-term treatment of asthma. The glutamine variant was associated with a marked improvement in AHR after treatment with inhaled corticosteroids (28). Interestingly, in the current study, this nonsynonymous variant demonstrated the greatest degree of transmission distortion to asthmatic offspring, with a transmitted:untransmitted ratio of approximately 3:2 for the glutamine allele. Because of the relatively small number of informative families ($n = 54$), this distortion did not reach statistical significance, and so it is not possible to claim this SNP as an asthma-susceptibility variant. Substantially larger cohorts would be needed to test this hypothesis.

This study is the fourth to characterize the genetic variation of the T-bet locus and to test this variation in asthmatic cohorts. Chung and colleagues tested seven SNPs and one major haplotype in a Korean hospital-based cohort of 550 patients with asthma and 170 control subjects and found no evidence for association with asthma, serum IgE, or skin-test reactivity (6). Ylikoski and colleagues evaluated 15 SNPs in 120 asthma families from a founder population in central eastern Finland, and similarly did not detect evidence of association with either asthma or serum IgE levels (7). Akahoshi and colleagues recently

TABLE 4. T-BET HAPLOTYPE BLOCK ASSOCIATIONS WITH AIRWAY RESPONSIVENESS IN WHITE CAMP FAMILIES

Haplotype	Frequency	n*	SNPs							z-Score	Biallelic p Value
			c.-1993	c.-1514	IVS1+7240	IVS4-4	IVS5-22	c.2122			
Combined block 1 and 2 meta-haplotypes (multiallelic p value = 0.02)											
H1/H4	0.571	243	T	T	T	C	C	G	-0.486	0.63	
H2/H5	0.179	126	C	C	T	C	T	G	1.156	0.25	
H1/H6	0.111	126	T	T	C	C	C	G	-1.999	0.04	
H3/H8	0.055	65	C	T	T	G	C	G	0.145	0.88	
H1/H7	0.043	6	T	T	T	C	C	A	2.213	0.03	
H3/H7	0.023	29	C	T	T	C	C	A	0.690	0.49	
Block 1 haplotypes (multiallelic p value = 0.59)											
H1	0.730	198	T	T					-0.868	0.39	
H2	0.178	150	C	C					1.335	0.18	
H3	0.088	119	C	T					-0.190	0.85	
Block 2 haplotypes (multiallelic p value = 0.04)											
H4	0.578	328			T	C	C	G	-1.236	0.22	
H5	0.183	199			T	C	T	G	0.974	0.33	
H6	0.112	149			C	C	C	G	-1.625	0.10	
H7	0.070	104			T	C	C	A	2.216	0.03	
H8	0.056	81			T	G	C	G	0.699	0.48	

For definition of abbreviations, see Table 3.

* n denotes number of informative families. Analysis was restricted to haplotypes that were observed in at least 10 informative families. A total of 15 meta-haplotypes (combinations of block 1 and 2 haplotypes) were observed, of which 9 were seen at frequencies less than 0.01. The T-C block 1 haplotype was observed at a frequency of 0.006, and a sixth block 2 haplotype (C-C-T-G) was observed at frequency of 0.006.

TABLE 5. BASELINE CHARACTERISTICS OF NORMATIVE AGING STUDY SUBJECTS GENOTYPED FOR T-BET SINGLE NUCLEOTIDE POLYMORPHISMS

Variable*	Median (interquartile range) or n (%)	
	Responders (n = 200)	Nonresponders (n = 436)
Age	62 (5–68)	59 (54–64)
History of asthma	66 (33%)	0 (0%)
FEV ₁ , L	2.68 (2.29–3.13)	3.45 (3.11–3.79)
FEV ₁ , % predicted	90% (76–101%)	108% (99.4–119%)
FEV ₁ /FVC ratio	0.734 (0.672–0.781)	0.806 (0.770–0.841)
Methacholine		
dose–response slope	3.26 (2.75–4.48)	0.45 (0.36–0.53)
Total serum IgE	51.5 (15.28–138.8)	24.9 (8.8–64.5)
Eosinophil count	229 (141–334)	158 (88–246)
Skin-test positive > 2 mm [†]	120 (60%)	204 (46.8%)
Packs per day	20 (0.1–45)	7.5 (0–28)
Smoking status		
Never	50 (25%)	157 (36%)
Past	40 (20%)	39 (8.9%)
Current	110 (55%)	240 (55%)

* Differences between responders and nonresponders significant at $p < 0.01$ for all variables.

[†] Skin-test positivity to at least one of the following allergens: ragweed, house dust, mixed trees, mixed grass.

demonstrate associations of the –1993C allele with aspirin-induced asthma in a Japanese case-control analysis. In that study, no association of this allele or three others was observed with either adult or childhood asthma not related to aspirin intolerance (8). No significant associations with –1993 were observed in our cohort, and we do not have detailed information regarding aspirin sensitivity in CAMP, preventing evaluation of the associations of –1993C with aspirin-induced asthma in our cohort. Our major finding was evidence of a relationship between multiple T-bet SNPs and AHR, a phenotype that was not evaluated in the other populations. We also found association with T-bet SNP c.–7947 and asthma. This variant is located approximately 8 kb upstream from the T-bet transcript and was not evaluated in the other studies. Because this variant is not in strong LD (in our cohort) with those SNPs that overlap between the four studies, it is unclear whether c.–7947 would have demonstrated association with asthma in the other populations. In addition, the strength of association between asthma and c.–7947 in our cohort is at best modest, and it is quite possible that this observation was observed by chance and is not biologically meaningful. Additional replication studies of this locus are warranted. One striking area of agreement across all studies is the high degree of sequence conservation across T-bet: only two nonsynonymous coding variants (His33Gln and Ile339Val) were found in the aggregate pool of 186 chromosomes surveyed in these four reports (48 Korean chromosomes, 46 Finnish chromosomes, 48 Japanese chromosomes, and 44 white chromosomes), and the bulk of observed variation mapped to regions of relatively low phylogenetic conservation. As suggested by Ylikoski (7), this restricted T-bet diversity likely reflects the important functions of T-bet for proper immune system development.

As with the two previous studies that assessed associations with IgE levels, we did not find evidence of a relationship between T-bet SNPs and serum IgE levels in either CAMP or NAS. The lack of any association with atopy-related phenotypes in four populations suggests that T-bet genetic variation does not appear to directly influence clinical markers of atopy such as total serum IgE or blood eosinophil levels. Given our understanding of T-bet's role in T-cell differentiation, these results are perhaps surprising and the associations with AHR all the

more intriguing: through what mechanisms could variation in T-bet alter airway responsiveness without affecting these atopic phenotypes? T-bet-deficient mice manifest AHR to methacholine, peribronchial eosinophilic inflammation, and chronic peribronchial fibrosis and remodeling (3), suggesting that the airway responsiveness-associated variants (or untyped variants in LD) somehow confer a T-bet-deficient state. This notion is further supported by the observations of reduced T-bet expression in CD4⁺ lymphocytes in asthmatic airways in humans (3). It has been shown that CD4⁺ lymphocytes in asthmatic airways have Th2-type cytokine profiles, even among patients with asthma who do not manifest classic atopic features (so-called intrinsic patients with asthma), suggesting that subtle Th2 skewing is present in most forms of asthma (29). These effects may be mediated by IL-13, which is expressed at higher levels in asthmatic bronchial mucosa (30) and mediates eosinophil recruitment, mucus hypersecretion, and AHR in murine models, even in the absence of other Th2 cytokines IL-4 and IL-5 (31, 32). In T-bet-deficient mice, local blockade of IL-13 by intranasal administration of IL-13 blocking antibody reduces airway inflammation, airway responsiveness, and subsequent airway remodeling, suggesting that IL-13 derived from T-bet-deficient activated CD4⁺ lymphocytes residing in the airways plays a central role in this process (33). Although it is conceivable that the functional consequences of human T-bet SNPs are restricted to the airway, it is more plausible that these variants have more subtle effects on Th1:Th2 balance that is not discernible by assessing clinical phenotypes such as serum IgE or blood eosinophil levels. Functional studies of these variants and studies correlating T-bet genotype with cytokine expression profiles would help resolve these questions. It is noteworthy that Akahoshi and colleagues recently provided *in vitro* data suggesting that the –1993C allele (i.e., the allele associated with aspirin-induced asthma) may confer increased T-bet expression (8).

In summary, we have demonstrated evidence of an association between T-bet polymorphism and the degree of AHR among white children with asthma, with modest evidence that these findings can be extended to other populations demonstrating airway responsiveness. Because associations were observed with SNPs that are not in tight LD with each other, it is unclear whether or not any of the identified SNPs are the truly functional variants, or whether these loci are in linkage disequilibrium with functional variants that have not been typed. It is also possible that the c.–7947 associations relate to effects on a neighboring gene, TBK1-binding protein 1, which is situated approximately 21 kb upstream from T-bet and has been implicated in tumor necrosis factor- α /nuclear factor- κ B signaling (34). Although this remains a possibility, the other associations of downstream T-bet SNPs with AHR, and our previous findings of pharmacogenetic effects of the T-bet H33Q variant, argue somewhat against this possibility. Although additional genetic studies of AHR may be useful to address this question, resolving this issue will likely require comprehensive functional evaluations of these variants.

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