

TOLL-like Receptor 10 Genetic Variation Is Associated with Asthma in Two Independent Samples

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TOLL-like receptor 10 (*TLR10*) is the most recently identified human homolog of the *Drosophila* TOLL protein. In humans, the TOLL-like receptors recognize pathogen-associated molecular patterns (PAMPs) as part of innate immune host defenses. Localized to chromosome 4p14, the specific ligands and functions of *TLR10* are currently unknown, although it is expressed in lung and in B-lymphocytes. *TLR10* is a potential asthma candidate gene because early life innate immune responses to ubiquitous inhaled allergens and PAMPs may influence asthma susceptibility. Resequencing in 47 subjects revealed a total of 78 single nucleotide polymorphisms (SNPs) (1 SNP per 106 bp) of which only 11 had been previously published. A significant association ($p < 0.02$) between two SNPs (c.+1031G>A, c.+2322A>G) and physician-diagnosed asthma was observed in a case control study (517 cases, 519 control subjects) of European American subjects nested within the Nurses' Health Study cohort. The association for these same two SNPs ($p \leq 0.015$) replicated in an independent family based cohort, where a measure of airway hyperresponsiveness (PC_{20}) was also associated ($p = 0.026$ for c.+1031G>A). Consistent association in two independent samples and association with an intermediate phenotype provides strong support for *TLR10* genetic variation contributing to asthma risk.

Keywords: asthma; single nucleotide polymorphisms; TOLL-like receptor 10

TOLL-like receptors (TLRs) are highly conserved homologs of the TOLL protein of *Drosophila*. In mammals these germ-line encoded receptors play a fundamental role in innate immunity by recognizing pathogen-associated molecular patterns (PAMPs) expressed on microorganisms and mediating the production of cytokines necessary for nonadaptive response and effective host defense. TLR-dependent signaling is also critical for the activation of adaptive responses, which require soluble factors and costimulatory molecules induced by pathogen-mediated TLR engagement. The human *TLR10* gene occupies 3,269 bases arranged in three exons on the short arm of chromosome 4 (4p14) and encodes an 811-amino acid protein, approximately 50% identical to TLR1 and to TLR6 (1). Like other members of the TLR family, *TLR10* contains a signal peptide, multiple ($n = 12$) leucine-rich repeats, a cysteine-rich domain, a transmembrane domain, and a cytoplasmic TOLL interleukin-1 receptor domain.

Although the ligand and the specific functions of human *TLR10* are not currently known, it is predominantly expressed in immune

cell-rich tissues, including spleen, lymph node, and lung (1). *TLR10* expression is barely detectable in naive human B cells, but is rapidly induced after B-cell receptor triggering (2).

Asthma is a common chronic lung disease, affecting more than 26.7 million people in the United States (3) and known to be associated with substantial heritable risk (4, 5). Asthma is primarily a disease of the airways, characterized by chronic inflammatory changes, reversible airflow obstruction, and increased responsiveness to a variety of stimuli including common allergens. Because the airways are regularly exposed to inhaled suspended environmental allergens and PAMPs, asthma susceptibility may be at least partially associated with disordered immune responses to common respirable environmental exposures (6). If this is true, then asthma susceptibility might also be associated with variation in genes encoding components of innate immunity (7), particularly those known to be expressed in lung tissue such as *TLR10*, making them biologically plausible candidate genes for asthma.

Although the current public database (dbSNP release 118) contains only 11 single nucleotide polymorphisms (SNPs) within the immediate region of the *TLR10* gene, we have previously reported relatively high levels of polymorphism in innate immunity genes (7). In the absence of any other published human surveys of the gene, we resequenced *TLR10* in 47 anonymous samples from two U.S. populations. We tested the hypothesis that genetic variation in *TLR10* contributes to asthma susceptibility in a case-control genetic association study nested within a well established cohort study and, for replication, in an independent family-based study of childhood asthma.

METHODS

Resequencing Samples and Processing

TLR10 was resequenced in 47 samples (24 African American, 23 European American; Coriell Institute, Camden, NJ) and SNPs were named relative to the start of the *TLR10* coding sequences (CDS) in Genbank accession NT_016297.15, so c.+1031G>A refers to a SNP located 1,031 bases 3' from the first base of the *TLR10* coding sequence, with G as the common base and A as the minor allele. Where available, dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) identifiers are also given (e.g., rs4129009). Detailed protocols are available at the Innate Immunity NHLBI Programs in Genomics Applications website (<http://innateimmunity.net>).

Association Study Design

Three European American haplotypes seen more than once could be distinguished using two haplotype-tagging SNPs (htSNPs) (8), identified with the BEST algorithm (9). We genotyped these in individuals with asthma and in control subjects from the Nurses Health Study (10) (NHS). Cases ($n = 517$) were self-reported lifelong nonsmokers with physician-diagnosed asthma. Nonsmoking control subjects without asthma ($n = 519$) were matched on age.

Family-based Analysis Sample and DNA

The Childhood Asthma Management Program (CAMP) was a clinical trial in children with mild to moderate asthma (11, 12). Blood samples were collected as part of the CAMP protocol at each local center.

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Consent and Human Subjects Approvals

Informed consent and assent were obtained from all CAMP and NHS study participants. Both studies were approved by the Institutional Review Board of the Brigham and Women's Hospital.

Family-based Analysis Phenotypes

Pulmonary function tests were performed by CAMP-trained technicians using a volume displacement spirometer in accordance with ATS criteria (13, 14). The concentration of methacholine (PC_{20}) (in mg/ml) that causes a 20% decline in FEV_1 was measured (11) for each subject.

SNP Genotyping

All genotyping used multiplexed single-base extension with separation on a Sequenom machine (Sequenom, Inc., San Diego, CA). Genotyping of approximately 10% of samples was performed twice as part of the standard laboratory quality control procedures. Detailed protocols are available from the Innate Immunity PGA Website (<http://innateimmunity.net>).

Analysis

Genotype Hardy-Weinberg equilibrium (HWE) was tested using an exact method (15). Pairwise Linkage Disequilibrium (LD) was calculated (16) as r^2 (17). Fisher's Exact test (18) was used for all contingency table inference. Haplotypes were inferred using Bayesian methods (19, 20), separately for each sample. Only SNPs at or above 10% MAF were considered for haplotypes or association, because our sample size gave inadequate power for SNPs below this frequency.

A total of 25 Mendelian inconsistent (21) transmissions, restricted to 8 specific pedigrees (inconsistencies per SNP ranged from 1 to 4) were identified, and all parental and offspring genotypes for these transmissions were removed from the CAMP family-based analysis. The primary family-based analysis was for association of individual SNPs with asthma using the Family Based Association Test (FBAT) program (22). Software that estimates statistical power (PBAT) for FBAT tests (23) was used to estimate the conditional power of the FBAT statistic under additive, dominant, and recessive models (24), and only the model with the highest average power was tested. A generalized principal component (PC) test designed for repeated measurements, combining all 6 PC_{20} measurements for each subject into a single FBAT test (24), was used to perform a CAMP cohort secondary analysis for each SNP.

RESULTS

A total of 78 polymorphisms were identified at a minor allele frequency (MAF) greater than 1% among the 47 individual samples resequenced (Table 1) in 8,278 bp. Ten of the 11 polymorphisms found in the public database (dbSNP, build 118) were confirmed in one or both samples (Table 1), but one (rs4429735), an SNP predicted from *in silico* analysis, was not detected. All polymorphisms had genotype counts compatible with Hardy-Weinberg equilibrium (HWE) within each ethnic group (all $p > 0.07$) using an exact test (15). Twenty-three polymorphisms were seen only in African-American samples and five were seen only

TABLE 1. TLR10 SNP LOCATIONS AND ALLELE COUNTS IN RESEQUENCED EUROPEAN- (n = 23, 46 CHROMOSOMES) AND AFRICAN-AMERICAN (n = 24, 48 CHROMOSOMES) SAMPLES

dbSNP (build 118) ID	Marker	Coding Change	Minor Allele and Frequency		
			Base	African American	European American
rs4130727	c.-3902T>C		C	0.17	0.09
	c.-3689A>T		T	0.02	0.00
	c.-3617G>A		A	0.20	0.02
	c.-3563A>G		G	0.02	0.00
	c.-3510A>G		G	0.22	0.02
	c.-3446T>C		C	0.07	0.00
	c.-3436G>A		A	0.25	0.02
	c.-3329T>C		C	0.25	0.02
	c.-3286C>A		A	0.02	0.00
	c.-3260A>G		G	0.04	0.15
	c.-3235A>C		C	0.02	0.00
	c.-3220A>C		C	0.21	0.02
	c.-3070G>A		A	0.04	0.00
	c.-3043C>A		A	0.21	0.02
	c.-3042G>A		A	0.02	0.00
	c.-2801T>G		G	0.24	0.02
	c.-2778T>A		A	0.02	0.00
	c.-2769G>C		C	0.22	0.02
	c.-2698C>A		A	0.24	0.02
	c.-2688C>G		G	0.22	0.02
	c.-2592T>—		—	0.02	0.00
	c.-2411G>T		T	0.07	0.00
	c.-2403A>T		T	0.07	0.00
	c.-2302C>T		T	0.09	0.00
	c.-2301G>A		A	0.04	0.14
	c.-2299C>T		T	0.21	0.02
	c.-2243C>T		T	0.00	0.02
	c.-2157T>C		C	0.02	0.00
	c.-2023T>C		C	0.06	0.00
	c.-2002A>T		T	0.21	0.02
rs7694115	c.-1883T>C		C	0.48	0.26
	c.-1880T>C		C	0.22	0.02
rs6852188	c.-1769T>A		A	0.22	0.02
	c.-1692C>T		T	0.04	0.12
rs6825115	c.-1622C>T		T	0.21	0.02
rs6856864	c.-1621A>G		G	0.20	0.02
rs6856416	c.-1362A>G		G	0.21	0.02

TABLE 1. (Continued)

dbSNP (build 118) ID	Marker	Coding Change	Minor Allele and Frequency		
			Base	African American	European American
rs6824319	c.-1236C>T		T	0.11	0.00
	c.-1163+>—		—	0.17	0.02
	c.-1044G>A		A	0.00	0.04
	c.-1030C>T		T	0.20	0.02
	c.-996A>G		G	0.07	0.02
	c.-992T>A		A	0.04	0.15
	c.-664G>A		A	0.21	0.02
	c.-558A>—		—	0.02	0.00
rs4274855	c.-309A>T		T	0.21	0.02
	c.-306T>G		G	0.21	0.02
	c.-260G>A		A	0.04	0.15
	c.-172C>T		T	0.21	0.02
	c.-73C>A		A	0.04	0.00
	c.-25A>C		C	0.42	0.11
	c.+38T>A	Ile-Ile	A	0.42	0.11
	c.+152G>A	Thr-Thr	A	0.21	0.02
	c.+269A>G	Lys-Lys	G	0.06	0.00
	c.+486G>T	Ala-Ser	T	0.08	0.02
	c.+499T>C	Leu-Pro	C	0.00	0.02
	c.+720A>C	Asn-His	C	0.46	0.26
	c.+891G>A	Val-Ile	A	0.08	0.02
	c.+908A>G	Lys-Lys	G	0.21	0.09
	c.+976T>C	Met-Thr	C	0.06	0.02
	c.+1031G>T	Pro-Pro	T	0.25	0.17
	c.+1082A>G	Thr-Thr	G	0.02	0.00
	c.+1104A>C	Ile-Leu	C	0.46	0.26
	c.+1141G>A	Gly-Asp	A	0.04	0.00
	c.+1404C>G	Arg-Gly	G	0.02	0.02
	c.+1417T>C	Ile-Thr	C	0.02	0.07
	c.+1572C>T	Arg-Trp	T	0.00	0.04
	c.+1806C>T	Leu-Leu	T	0.06	0.00
	c.+2171T>C	His-His	C	0.25	0.17
	c.+2206A>G	Tyr-Cys	G	0.02	0.00
	c.+2322A>G	Ile-Val	G	0.04	0.15
rs4129009	c.+2395G>A	Arg-Gly	A	0.00	0.02
rs4129008	c.+2426T>C	Asp-Asp	C	0.25	0.17
	c.+2708A>G		G	0.25	0.17
	c.+2722T>G		G	0.04	0.15
	c.+2729C>A		A	0.56	0.24
	c.+2782T>C		C	0.02	0.00
	c.+2822C>T		T	0.08	0.04

Markers are identified relative to the TLR10 CDS in the reference genomic sequence NT_016297.15. Insertion-deletion polymorphisms are shown with — for the deleted allele. The insertion-deletion polymorphism at c.-1163 was a 15-base deletion with a sequence of CTCTGTCTACTGCT.

in the European-American samples. Twenty-two SNPs (13 non-synonymous) were detected in coding regions (Table 1).

Forty-four SNPs were observed at 10% or greater MAF in one or both sequenced samples. One SNP seen at 11% in African-American samples was not detected in European-American samples. The 15 base deletion polymorphism at c.-1163 was observed eight times (17.4%) in African Americans but was only seen once (2%) in a European-American subject.

Pairwise linkage disequilibrium (LD) for SNPs at 10% or greater MAF expressed as r^2 (16, 25) is shown in Figure 1 for each ethnic group separately. In the European sample, the region is characterized by uniformly strong LD with the exception of the two SNPs at c.-25 and c.+38, both of which have relatively low MAF (11%), which are in strong LD with each other but in weak LD with the other SNPs. The African-American sample had many more common SNPs, particularly in the 5' flanking region and strong LD throughout the region sequenced, with the exception of the SNP at c.+908, compared with the European-American sample.

Haplotypes for all SNPs at 10% or greater MAF in either

European-American or African-American samples, inferred for each group separately, are shown in Table 2. The European-American sample had three haplotypes accounting for 93% of all chromosomes, compared with 67% in the most common three haplotypes among the African-American sample. The three haplotypes seen more than once in European Americans can be unambiguously distinguished using two htSNPs (8) at -25 and 1,031. These two htSNPs and two other SNPs (c.+2322 and c.+2729, each in strong LD with one of the htSNPs for redundancy) were optimized for genotyping.

One SNP (c.+2729) was discarded because of poor quality control results during optimization. The remaining three SNPs were genotyped in subjects with asthma and in control subjects nested within a well established cohort study (26). One SNP showed no significant association with asthma (c.-25) and was in HWE among both cases and control subjects. The other two SNPs were in proportions compatible with HWE among control subjects but not in cases ($p = 0.012$ for c.+1031 and $p = 0.005$ for c.+2322). Genotype distributions for the two SNPs in strong LD were significantly associated with asthma (Fisher's exact test,

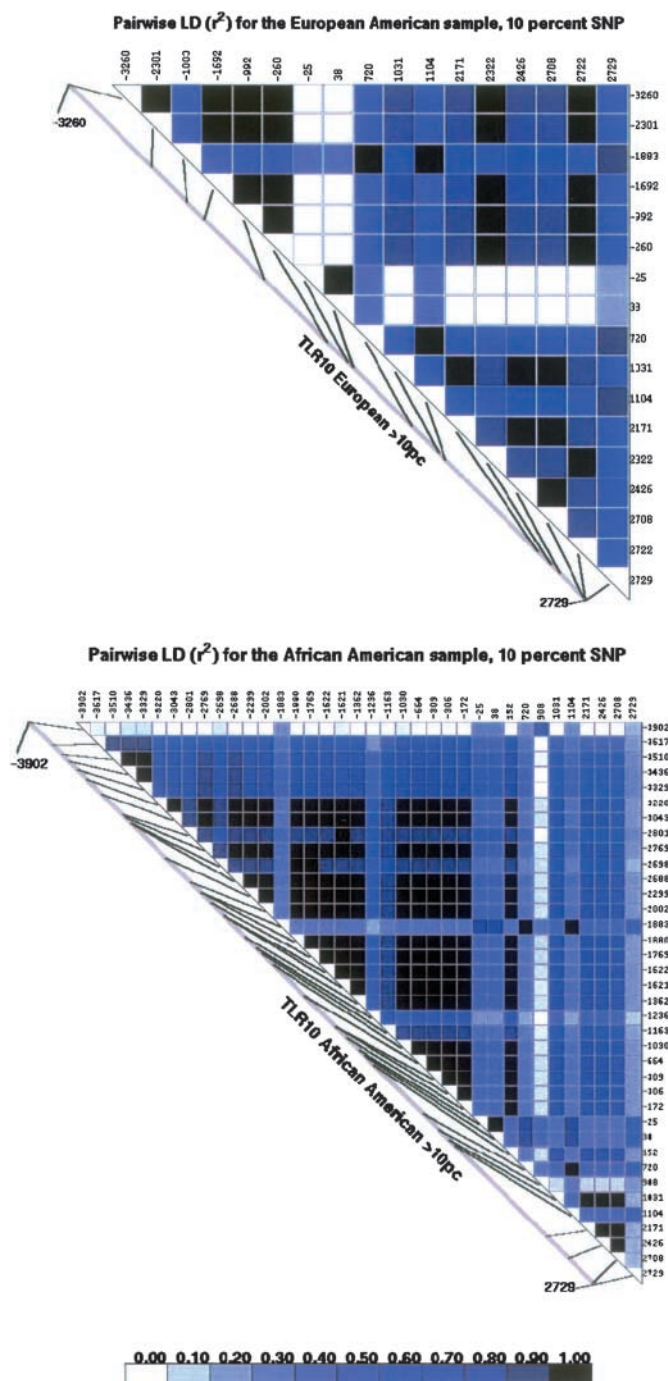


Figure 1. Pairwise linkage disequilibrium (LD) (expressed as r^2) from *TLR10* resequencing data for SNPs > 10% MAF for European American and African American samples separately.

$p = 0.02$) as shown for c.+2322 (rs4129009) in Table 3 and for the SNP at c.+1031 in Table 4.

A total of 10 SNPs were selected for genotyping in an independent sample of nuclear families that include children with asthma. There were too few informative families ($n = 2$) for reliable inference on transmission on one of these (c.+1417), so it was discarded from further analysis. Conditional power calculations using PBAT indicated that a recessive genetic model provided the greatest statistical power for our analyses. Using the FBAT and a recessive model, minor alleles were significantly undertransmitted to affected children for seven of these SNPs,

as shown in Table 5. Log transformed PC_{20} , a measure of airway hyperresponsiveness and a reliable intermediate phenotype for asthma, was significantly associated with SNPs at c.-3260, c.-260, c.+1031, and c.+2322, as shown in Table 6.

DISCUSSION

Like many other innate immunity genes studied to date, *TLR10* is highly polymorphic (7). Resequenced DNA samples from 47 unrelated subjects in two self-identified U.S. ethnic groups revealed a total of 78 SNPs and insertion-deletion polymorphisms in the 4p14 genomic region containing the gene. Averaging about one polymorphism for each 100 bases sequenced, this is about three times higher than rates of 1 SNP per 333 bases recently estimated from very deep resequencing (27), and an order of magnitude greater than widely quoted older estimates (28) of 1 SNP per 1,000 bases. Five SNPs were detected only in European-American samples and another 23 were detected only in African-American samples. These 28 population-specific variants were seen at relatively low frequency, whereas the SNPs seen in both samples tended to be at higher frequencies, as has been described in other multiethnic studies (29).

Although the *TLR10* region displayed extremely high rates of polymorphism, there were also high levels of pairwise LD and relatively low haplotype diversity. This is reflected in the finding that three haplotypes accounted for the vast majority of both European-American and African-American chromosomes. Limited haplotype diversity over short "blocks" (30) permits a relatively small number of SNPs to "tag" relatively large haplotypes, with substantial decreases in the costs of genotyping (8, 9). The small region of relatively low pairwise LD near the start of the first exon may be partially explained by the relatively low allele frequency of the SNPs involved, because pairwise LD values are lower when there are large differences in allele frequencies (31). Relatively recent mutation on one of the less common background haplotypes might give rise to a similar pattern.

The three common European-American chromosomes could be unambiguously distinguished using two htSNPs (8, 9), which were genotyped in a case-control association study for asthma, a disease thought to have a potential relationship to innate immunity. Genotypes for both of the two SNPs in strong LD with each other were not in proportions compatible with HWE among cases, although they were among control subjects, suggesting that laboratory errors such as miscalled genotypes or sample contamination (which can readily introduce spurious departures from HWE) are unlikely to account for the finding. Departure from HWE among cases has been suggested as a method for screening markers for disease association (32) and has been noted in other positive association studies (33). Moreover, these two SNP genotypes were found to be associated with asthma ($p = 0.02$) with an excess of minor homozygotes among the cases. One of these (c.+1031G>A) is a synonymous (Proline to Proline) coding SNP; the other (c.+2322A>G, rs4129009) introduces a change from Isoleucine to Valine at amino acid position 775 in the *TLR10* protein (Swiss-Prot Q9BXR5), which is in the cytoplasmic *TOLL* Interleukin-1 receptor domain of the protein. A change in the protein in this region could provide a biologically plausible basis for an effect on *TLR10* signaling through altered IL1 binding. However, because the whole region is characterized by strong LD as shown in Figure 1, it is also possible that the moderate association we detected in the case-control study might be causally related to a nearby variation that we did not genotype (25).

Both of the SNPs (c.+1031G>A and c.+2322A>G) significantly associated with disease in the case-control study were also significantly associated with asthma in an analysis among an

TABLE 2. HAPLOTYPES INFERRED USING THE PHASE PROGRAM (19) FOR SNPs WITH 10% OR GREATER MINOR ALLELE FREQUENCY IN EITHER RESEQUENCED SAMPLE

Haplotype	All Combined		African American		European American	
	N	Percent	N	Percent	N	Percent
TGAGTAACTGCCGCATTTC AAC+CTGATGCATGAAGATATATC	52	55.3%	19	39.6%	33	71.7%
CGAGTAACTGCCGCACCTTCCAAC+CTGATGCCAGCGGCTATATA	10	10.6%	7	14.6%	3	6.5%
TGAGTGAAGTCCACACTTTCCAAC+CAGATACATGCATCCGCGGA	8	8.5%	1	2.1%	7	15.2%
TGAGTAACTGCCGCATTTC AAC+CTGATGCATGAAGATATATA	6	6.4%	6	12.5%	0	0.0%
TAGACACAGCAGGTTCCACTGGT-TTATGGTCAACATCCACGTA	5	5.3%	5	10.4%	0	0.0%
TAGACACAGCAGGTTCCACTGGC-TTATGGTCAACATCCACGTA	3	3.2%	3	6.2%	0	0.0%
TAGACAACTGCCGCACCTTCCAAC+CTGATGCCAGCGGCTATATA	2	2.1%	2	4.2%	0	0.0%
TGGACACAGCAGGTTCCACTGGT-TTATGGTCAACATCCACGTA	1	1.1%	1	2.1%	0	0.0%
TGAGTGAAGTCCACACTTTCCAAC+CAGATACATGCATCCGCGGG	1	1.1%	1	2.1%	0	0.0%
TGAGTAACTGCCGCATTTC AAC+CTGATGCATGAAGATATATC	1	1.1%	1	2.1%	0	0.0%
TGAGTAAATCAGGTACTTCTAAC-CTGATGCCAGCAGCCACGTA	1	1.1%	0	0.0%	1	2.2%
TAGACACCGCGCGCTTCCACGGC+TTATGGTATAAATATATATC	1	1.1%	0	0.0%	1	2.2%
TAGACACAGCAGGTTCCACTGGC+TTATGGTCAACATCCACGTA	1	1.1%	1	2.1%	0	0.0%
CGAGTAACTGCCGCATTTC AAC+CTGATGCCAGCGGCTATATC	1	1.1%	0	0.0%	1	2.2%
CGAGTAACTGCCGCACCTTCCAAC+CTGATGCCAGCGGCTATATA	1	1.1%	1	2.1%	0	0.0%
Totals	94	100.0%	48	100.0%	46	100.0%

The markers (see Table 1) for 44 SNP forming the haplotypes shown in Table 2 are as follows: c.-3902, c.-3617, c.-3510, c.-3436, c.-3329, c.-3260, c.-3220, c.-3043, c.-2801, c.-2769, c.-2698, c.-2688, c.-2301, c.-2299, c.-2002, c.-1883, c.-1880, c.-1769, c.-1692, c.-1622, c.-1621, c.-1362, c.-1236, c.-1163, c.-1030, c.-992, c.-664, c.-309, c.-306, c.-260, c.-172, c.-25, c.+38, c.+152, c.+720, c.+908, c.+1031, c.+1104, c.+2171, c.+2322, c.+2426, c.+2708, c.+2722, c.+2729.

independent sample of parent-child trios ascertained for asthma in the children (13, 14). In total, the patterns of transmission of seven of nine SNPs genotyped in the family-based sample were significantly associated with asthma at $p < 0.05$. The c.-25A>C SNP, which was not significant in the case-control analysis, was similarly not significant in the family-based analysis, whereas the SNP at c.+720A>C was of borderline statistical significance ($p = 0.051$) in the family-based analysis.

Genetic risk factors for asthma have recently been a subject of considerable interest (34). Specific SNP associations reported in *ADAM33* (35) have not consistently replicated among a number of subsequent, independent studies (36-38). More recently, positional cloning of asthma susceptibility loci in a founder population, including an orphan G protein-coupled receptor gene on the short arm of chromosome 7 has been reported, and the association is supported with both consistent independent replication and animal model data (39). Although many other asthma susceptibility loci have been reported, only a few have been replicated in independent samples (40).

Inadequate sample size, population stratification, and multiple statistical comparisons are important considerations in interpreting complex genetic disease association study findings (41). Although strict control of Type I error is possible over multiple statistical tests, it leads to substantial risk of false negative findings through loss of statistical power (42), and commonly employed methods such as the Bonferroni correction are invalid when tests are highly correlated (42), as is the case with multiple

SNPs in LD with each other. More recently developed methods that control the False Discovery Rate (43) help to quantify the reliability of apparently significant statistical findings over many tests. However, replication in an independent sample provides strong evidence of potential generalizability, because subsequent replication of a false positive finding in an independent sample is arguably an independent event, with an expected probability given by the product of the two independent Type I error p values or 0.0025.

We do not believe that our findings can be explained by multiple statistical testing. Our strategy was to screen htSNPs, determined by inferring haplotypes from SNP discovery resequencing data, for association in the NHS. Only three SNPs were successfully genotyped in the NHS, of which two were significantly associated in three statistical tests. Both significant NHS SNPs were also significant in the CAMP replication data, whereas the nonsignificant NHS SNP was confirmed to be nonsignificant in the replication. A number of additional markers only genotyped in the replication data were in LD with the associated SNPs and were also significantly associated with asthma, but these were confirmatory and their p values were not used to generate hypotheses.

This study has a number of important strengths in terms of design, sample size, and replication. Resequencing of the region containing the *TLR10* gene in samples from two North American population samples revealed a large number of previously undetected variations and enabled SNP selection for case-control genotyping based on an understanding of the haplotype and LD distributions in the target population. Control subjects were matched

TABLE 3. GENOTYPE COUNTS (PERCENT) FOR c.+2322A>G (RS4129009) AMONG NHS ASTHMA CASES AND CONTROL SUBJECTS

	AA	AG	GG	Total
Control	344 (67.1%)	157 (30.6%)	12 (2.3%)	513
Case	346 (68.5%)	132 (26.1%)	27 (5.4%)	505
Total	690 (67.8%)	289 (28.4%)	39 (3.8%)	1018

Genotypes were missing for 6 control subjects and 12 cases. Fisher's exact test, $p = 0.0196$.

TABLE 4. GENOTYPE COUNTS (PERCENT) FOR c.+1031G>T AMONG NHS ASTHMA CASES AND CONTROL SUBJECTS

	GG	GT	TT	Total
Control	316 (61.5%)	180 (35.0%)	18 (3.5%)	514
Case	335 (66.2%)	142 (28.1%)	29 (5.7%)	506
Total	651 (63.8%)	322 (32.6%)	47 (4.6%)	1020

Genotypes were missing for 5 control subjects and 11 cases. Fisher's exact test, $p = 0.0235$.

TABLE 5. FBAT RESULTS FOR ASTHMA AFFECTATION, RECESSIVE MODEL, ALL CAMP FAMILIES

Marker	Allele	Allele Freq.	Informative Families	S	E(S)	Var(S)	Z Score	p Value
c.-3260A>G	G	0.16	55	11	18.3	12.19	-2.07	0.037
c.-260G>A	A	0.16	56	11	18.5	12.38	-2.13	0.033
c.-25A>C	C	0.21	87	32	31.5	19.75	0.11	0.910
c.+720A>C	C	0.37	199	66	79.3	46.31	-1.94	0.051
c.+1031G>T	T	0.22	90	17	31.5	20.00	-3.24	0.001
c.+1104A>C	C	0.37	194	64	78.0	45.50	-2.07	0.037
c.+2171T>C	C	0.22	87	18	29.3	19.44	-2.55	0.010
c.+2322A>G	G	0.16	53	10	18.3	11.56	-2.42	0.015
c.+2426C>T	T	0.22	83	16	29.3	18.56	-3.07	0.002

Note that p values for the minor allele only are shown (p values for common alleles were all > 0.15).

Definition of Abbreviations: E(S) = expected score under the null hypothesis; S = score value from the FBAT test; Var(S) = score variance.

to cases from within a long-established cohort study, so the findings are unlikely to be confounded by differences in allele frequencies between cases and control subjects due to differences in genetic origin or other cause of population stratification (44). The confirmatory family-based replication study was conducted in an extremely well characterized sample of substantial size, using methods robust to population stratification (45). Significant associations with *TLR10* SNPs were detected in both children and in adults. Association of *TLR10* variation with an objective measure of airways hyperresponsiveness and association with a nonsynonymous coding SNP in the cytoplasmic domain adds additional biological plausibility to the findings.

In the case-control adult sample, the rare homozygous genotype was overrepresented in cases for both significant SNPs, whereas in the recessive model for childhood asthma in the family-based tests, the rare allele was significantly undertransmitted, so the disease association signal we have detected in the *TLR10* locus might have arisen through different mechanisms in the adults compared with the children, as has been previously postulated for CD14 genetic variation (46). Although the association study samples reported here are reasonably large compared with many similar studies, we only tested relatively common SNPs because sample size calculations suggested that we had insufficient power to detect an effect from SNPs below 10% MAF. Replication of a significant association in two independent samples using methods appropriate to the two different study designs seems very unlikely to have arisen by chance and suggests that the locus association is real and potentially generalizable, although the causal allele may not yet have been identified.

The ligands, functions of, and the interactions between the 10 mammalian TLRs described to date are gradually being

unraveled (47). Some TLRs appear to recognize a variety of microbial structures (47) such as *TLR4* and bacterial lipopolysaccharides, *TLR2* and lipoproteins, and *TLR9* and unmethylated bacterial CpG motifs; others appear to be more specific (47), such as *TLR5* and bacterial flagellins, *TLR2* and peptidoglycans (48), and *TLR3* and viral double-stranded RNA. Engagement of a TLR ligand is followed by a cascade of innate immune responses including antigen presentation, costimulatory molecule expression on cell surfaces, and cytokine synthesis and release, ultimately leading to adaptive immunologic responses orchestrated by both T and B lymphocytes (47).

Among leukocytes, expression of *TLR10* mRNA appears to be restricted to B lymphocytes and is increased following engagement of B cell antigen-receptor complex or polyclonal B cell activators such as CpG DNA (49). Intense early life CpG DNA exposure has been postulated as one possible explanation for low asthma rates observed among children raised on European farms (50), and it has been suggested that the recent large increases in atopic disease prevalence (including asthma) may be associated with diminished early life exposure to microbial components (51), providing another potential mechanism by which variation in innate immunity genes could influence asthma susceptibility.

Although little detail is known about the precise role of *TLR10*, it is expressed in lymphoid tissue, B lymphocytes and notably, in lung tissue, where involvement in the innate immune responses of the lung to common respirable exposures such as allergens and PAMPs provides a potential biological basis for the replicated association between *TLR10* genetic variation and asthma and an association with an important intermediate asthma phenotype, reported here.

Conflict of Interest Statement: R.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.A.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; C.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.K.S. has received a grant of approximately \$250,000 per year from GlaxoSmithKline to study COPD genetics between 2001 and 2004; D.J.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; W.J.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; F.D.M. received \$13,100 in 2002, \$3,000 in 2003, and \$2,500 in 2004 from Merck for serving as a member of the Merck Scientific Advisory Board, and received \$1,500 from GlaxoSmithKline for presenting at a sponsored event in 2003, and as a member of the AstraZeneca Speakers Bureau has received \$10,000 in 2002 and \$2,500 in 2003 for lectures; S.T.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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TABLE 6. FBAT PRINCIPAL COMPONENTS TESTS FOR ASSOCIATION WITH AIRWAYS HYPERRESPONSIVENESS (LOGARITHMIC TRANSFORMED PC₂₀) ASSUMING A RECESSIVE MODEL

Marker	Allele	Allele Frequency	Informative Families	p Value
c.-3260A>G	G	0.16	55	0.050
c.-260G>A	A	0.16	56	0.037
c.-25A>C	C	0.21	87	0.857
c.+720A>C	C	0.37	199	0.092
c.+1031G>T	T	0.22	90	0.026
c.+1104A>C	C	0.37	194	0.102
c.+2171T>C	C	0.22	87	0.254
c.+2322A>G	G	0.16	53	0.039
c.+2426C>T	T	0.22	83	0.446

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