

HLX1 gene variants influence the development of childhood asthma

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Background: Major transcription factors controlling T_H1 and T_H2 development, such as T-box transcription factor and GATA3, might be centrally involved in asthma and atopic diseases. Only recently, the homeobox transcription factor H.20-like homeobox 1 (HLX1), interacting closely with T-box transcription factor, has been identified as an important regulator of T_H1 differentiation and suppressor of T_H2 commitment.

Objective: We investigated whether genetic variations in the *HLX1* gene exist and whether these could affect the development of childhood asthma.

Methods: The *HLX1* gene was resequenced in 80 chromosomes. Associations between identified polymorphisms, asthma, and atopic diseases were investigated in German children (total n = 3099) from the cross-sectional International Study of Asthma and Allergy in Childhood phase II. Functional properties of polymorphisms were studied by using luciferase reporter gene assays and electrophoretic mobility shift assays in T cells. All statistical analyses were performed with SAS/Genetics software (SAS Institute, Inc, Cary, NC).

Results: Nineteen polymorphisms were identified in the *HLX1* gene, and 2 tagging single nucleotide polymorphisms representing 7 polymorphisms were associated with childhood asthma in our study population. Two promoter polymorphisms, C-1407T and C-742G, contained in 1 tagging block were

associated with asthma (odds ratio, 1.44; 95% CI, 1.11-1.86; *P* = .0061), significantly decrease promoter transactivation, and disrupt specificity protein–transcription factor binding in *in vitro* experiments.

Conclusions: Our data suggest that polymorphisms in the *HLX1* gene increase the risk for childhood asthma. On the cellular level, altered binding of specificity protein–transcription factors to the *HLX1* promoter and subsequent changes in *HLX1* gene expression might contribute to these effects. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: HLX1, asthma, association study, genetic analysis, functional promoter analysis

Asthma and allergy are common diseases during childhood, but the underlying mechanisms are still not well understood. However, the involvement of T cell–regulated inflammation and T_H2-associated cytokine expression in the development and perpetuation of these diseases is undisputed.^{1,2} Signature transcription factors, such as the T cell–specific T-box transcription factor (T-bet) and the GATA binding protein 3 (GATA3), control T_H1 and T_H2 development from naive T_H cells and might thus be centrally involved in the development of allergy and asthma. Although GATA3 induces T_H2 development associated with the secretion of IL-4, IL-5, and IL-13,³ T-bet is responsible for the activation and maintenance of T_H1 development by inducing IFN- γ production.⁴ However, T-bet is only capable of inducing maximal IFN- γ release in cooperation with a second transcription factor, the homeobox transcription factor HLX1 (H.20-like homeobox 1), which itself is regulated by T-bet.⁵ In combination, T-bet and HLX1 actively suppress T_H2 commitment. HLX1 downregulates the IL-4 receptor α expression in naive CD4 T cells.⁶ Furthermore, T-bet and HLX1 have the unique ability to revert T_H2 cell commitment of T_H cells already expressing T_H2 cytokines.⁵

Thus it was speculated that genetic alterations in T_H1/T_H2 signature transcription factors might lead to a deregulation of immune responses and have considerable effect on the development of atopic diseases. Indeed, results of recent studies had identified common variants in the gene encoding T-bet that were associated with asthma in some,⁷⁻⁹ but not all, studies.^{10,11} Considering the close interaction between T-bet and HLX1, we hypothesized that genetic alterations in *HLX1* might also contribute to the pathogenesis of asthma. Therefore we investigated whether genetic variations in the *HLX1* gene exist that could affect the development of allergic diseases and asthma in a cross-sectional study population of German children (total n = 3099).

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Supported by National Genome Research Network (NGFN) research grant NGFN 01GS 0429, the NUW-S23T16 project (to P.R.), and the German Research Foundation as part of the Transregional Collaborative Research Program TR22 "Allergic Immune Responses of the Lung," grant A15. Genotyping was performed in the Genome Analysis Center (GAC) of the Helmholtz Zentrum Munich.

Disclosure of potential conflict of interest: P. Rosenstiel is a consultant for UCB and received grant support from Applied Biosystems. S. Schreiber receives grant support from the Deutsche Forschungsgemeinschaft and the Federal Ministry of Education and Research and has provided legal consultation for Abbott, Centocor, Chemocentryx, Elan, Genentech, Schering-Plough, and UCB Pharma. M. Kabesch received grant support from the European Union, the Federal Ministry of Education and Research, and the Deutsche Forschungsgemeinschaft. The rest of the authors have declared that they have no conflict of interest.

Received for publication April 6, 2008; revised September 8, 2008; accepted for publication September 25, 2008.

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0091-6749/\$34.00

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doi:10.1016/j.jaci.2008.09.047

Abbreviations used

HLX1: H.20-like homeobox 1
EMSA: Electrophoretic mobility shift assay
LD: Linkage disequilibrium
MAF: Minor allele frequency
OR: Odds ratio
SNP: Single nucleotide polymorphism
SP: Specificity protein
T-bet: T-box transcription factor
UTR: Untranslated region

METHODS**Population description for association studies**

The cross-sectional International Study of Asthma and Allergy in Childhood phase II was performed with the same standardized and validated study tools in Munich and Dresden.¹² The prevalence of asthma and allergies in 5629 schoolchildren ages 9 to 11 years was assessed based on self-administered questionnaires and objective measurements, such as lung function tests, skin prick tests, and serum IgE measurements. Descriptive demographics are shown in Table E1 (available in this article's Online Repository at www.jacionline.org).

Children whose parents reported a physician's diagnosis of asthma (at least once) or of spastic or asthmatic bronchitis (at least twice) in a self-administered questionnaire were classified as having asthma. Children's classification of hay fever or atopic dermatitis was based on a parental report of a physician's diagnosis of the respective diseases.

The sensitivity to 6 common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, and mixed grass and tree pollen) was assessed by means of skin prick testing with standardized extracts and lancets (ALK-Abelló, Hørsholm, Denmark). A child was considered atopic if a wheal reaction of 3 mm or greater occurred to 1 or more specific allergens after subtraction of the negative control's wheal size. Total serum IgE levels were measured by using the Immulite System (DPC Biemann, Bad Nauheim, Germany). All children of German origin with DNA and serum IgE measurements available were included in this analysis (n = 3099). Written informed consent was obtained from all parents of children included in the study. All study methods were approved by the appropriate ethics committees.

Resequencing and mutation screening

Twenty-two overlapping fragments were designed to investigate all exons and introns to cover 7919 bp of continuous sequence in and around the *HLX1* gene. Additionally, 1934 bp upstream (based on the first ATG of exon 1) and 1155 bp downstream of exon 4 were sequenced, thereby including the 5' untranslated region (UTR) and 3'UTR. Gene fragments of interest were amplified by means of PCR with specific primers (see Table E2 in this article's Online Repository at www.jacionline.org). Primers were designed with the NetPrimer software and obtained from Metabion GmbH (Planegg-Martinsried, Germany). PCR was carried out on standard cyclers (Eppendorf GmbH, Eppendorf, Germany) in a total volume of 50 μ L with 60 ng of genomic DNA, a primer concentration of 0.25 μ L each, 0.2 mmol/L of each deoxyribonucleoside triphosphate, and 0.8 U of Taq DNA Polymerase. PCR fragments were sequenced in 80 chromosomes from 40 unrelated randomly selected German adult volunteers by using an ABI 3730 sequencer (Applied Biosystems, Lincoln, Calif).

Genotyping

Genomic DNA was extracted from whole blood by using a standard salting-out method.¹³ To minimize the use of genomic DNA in further analyses, a modified primer extension preamplification¹⁴ or alternatively the GenomiPhi procedure (Amersham Biosciences, Freiburg, Germany) were applied for random DNA before amplification. DNA samples were genotyped by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

(Sequenom, Inc, San Diego, Calif). PCR assays and associated extension reactions were designed by using the SpectroDESIGNER software (Sequenom, Inc). All amplification and extension reaction conditions have been previously described,¹⁵ and specific primers are shown in Table E3 (available in this article's Online Repository at www.jacionline.org). Primer extension products were loaded onto a 384-element chip with a nanoliter pipetting system (SpectroCHIP, SpectroJet, Sequenom) and analyzed by using a MassARRAY mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The resulting mass spectra were analyzed for peak identification with the SpectroTYPER RT 2.0 software (Sequenom, Inc). For quality control, Hardy-Weinberg calculations were performed to ensure that each marker was within the expected allelic population equilibrium.

Luciferase assay

Jurkat T cells obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) were seeded in a 96-well plate at a density of 8×10^4 cells per well. The next day, cells were transfected with 35 ng of pGL3 plasmids, expressing a luciferase gene under the control of the *HLX1* promoter containing wild-type or polymorphic alleles at positions C-1407T, C-742G, or both together with 15 ng of pRL-TK *Renilla* reporter plasmid (Promega, Madison, Wis) for normalization of transfection efficiency and cell viability. Xtreme Gene Q was used as transfection reagent, according to the manufacturer's protocol (Roche, Mannheim, Germany). Eight hours after transfection, medium was exchanged with medium containing various concentrations of ionomycin (2 or 50 ng/mL) or medium alone. After 18 hours' incubation, cells were washed in PBS and lysed in 1 $\times$ passive lysis buffer (Promega). The dual luciferase assay was performed according to the manufacturer's instructions (Promega) by using a Genios Pro luminometer (Tecan, Bubenorf, Switzerland).

Electrophoretic mobility shift assay

Nuclear extract was prepared from 14×10^6 unstimulated Jurkat T cells, as previously described.¹⁶ Nuclear extracts were placed in aliquots and stored at -80°C . The protein concentration of nuclear extract was determined by using the bicinchoninic acid kit (Pierce, Rockford, Ill). Electrophoretic mobility shift assays (EMSAs) were performed by using 31-bp oligonucleotides carrying wild-type or polymorphic alleles at positions -1407 and -742 (C-1407T: 5'-ACCACGACGCTCCTC [C/T] TGCAACCAG GCCCAA-3'; C-742G: 5'-CCTGTGATCAAC [C/G] CTCCTTGCCCCGTGG-3'). As controls, oligonucleotides were used that contain a known specificity protein (SP) binding site (5'-ATTCGATCGGGGCGGGGCGAGC-3'¹⁷) or an unrelated binding site (position -1127/-1097 of the *IL13* 5'-flanking region: 5'-GGACTTCTAGGAAAACGAGGGAAGAGCAGGA-3'¹⁸). Annealing of the complementary oligonucleotide pairs was carried out in 10 mmol/L Tris Cl (pH 8), 1 mmol/L EDTA, and 100 mmol/L NaCl by boiling the samples for 5 minutes and cooling slowly to 37°C . Purified double-stranded probes (100 ng) were end-labeled with γ [³²P]-adenosine triphosphate (250 μ Ci) with T4 polynucleotide kinase (New England Biolabs, Ipswich, Mass). Free radioactivity was removed with mini Quick Spin Oligo Columns (Roche, Mannheim, Germany). For EMSA experiments, each binding reaction (20 μ L) contained 5 μ g of nuclear extract, 1 $\times$ binding buffer (10 mmol/L Tris Cl [pH 8], 1 mmol/L EDTA, 0.1 mmol/L β -mercaptoethanol), and 1 μ g/reaction poly(dI-dC)-poly(dI-dC) and was adjusted for a final concentration of 80 mmol/L NaCl and 4% glycerol. The respective ³²P-labeled protein/DNA complexes were resolved on a 5% polyacrylamide gel and run for 6 hours at 18 to 22 mA with 0.5 $\times$ Tris-borate-EDTA buffer at 4°C . All EMSA experiments were performed under the described conditions by using probes either carrying the wild-type or polymorphic alleles.

Statistical analysis

Linkage disequilibrium (LD) patterns were assessed by using Haploview software.¹⁹ Deviations from Hardy-Weinberg equilibrium were analyzed by using the χ^2 test, with expected frequencies derived from allele frequencies. Association between single nucleotide polymorphisms (SNPs) and dichotomous outcomes were tested by using χ^2 tests. As a reference group for every

TABLE I. Description of *HLX1* polymorphisms and their respective position within the gene, rs numbers, allele frequencies, LD, tagging SNPs, genotyping call rates in percentages, and the *P* value for a deviation from Hardy-Weinberg equilibrium in 1940 children from Dresden

SNPs	Position in relation to ATG*	Position in the gene structure	rs No.	MAF§	LD (r^2) with tagging SNP within block§	Block	Tagging SNP	Call R%	<i>P</i> value, HWE
1	A-1.633G	Promoter	rs41369048†	0.09	0.86	1			–
2	C-1.486G	Promoter	rs2738751	0.14	1	1	C-1.486G	99.6	.0303#
3	C-1.407T	Promoter	rs3806325	0.19	1	2	C-1.407T	97.5	.1869
4	C-796A	Promoter	rs41441946†	0.02	1	NA¶			
5	C-742G	Promoter	rs2184658	0.18	0.98	2		–	–
6	C-559T	Promoter	rs2807857	0.34	0.98	5		–	–
7	A-434G	5'UTR	rs2738752	0.32	0.83	4		–	–
8	T-429G	5'UTR	rs12730158	0.21	1	2		–	–
9	C-263A	5'UTR	rs2738754	0.25	0.87	5		–	–
10	T+346C‡	Exon 1	rs12141189	0.25	1	3	T+346C	97.4	.9735
11	C+1.562G	Intron 2	rs17597773	0.36	0.81	3		–	–
12	G+2.256A	Intron 2	rs2247213	0.33	1	4	G+2.256A	98.2	.4336
13	T+3.009C	Intron3	rs1317189	0.30	0.88	4		–	–
14	T+3.183A	Intron 3	rs868058	0.35	1	5	T+3.183A	99.3	<.0001#
15	C+3.958T	Intron 3	rs34180575†	0.19	0.98	2		–	–
16	C+4.431T‡	Exon 4	rs2738755	0.31	0.94	4		–	–
17	G+4.447A	Exon 4	rs3738182	0.19	0.98	2		–	–
18	C+4.524G‡	Exon 4	rs11578466	0.09	0.86	1		–	–
19	C+4.844T	3'UTR	rs2738756	0.09	0.86	1		–	–

Call R%, Genotyping call rates in percentage; HWE, Hardy-Weinberg equilibrium.

*Based on the National Center for Biotechnology Information GenBank sequence (accession no. AF217621).

†SNP previously not described in dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). All 3 variations have been submitted to the National Center for Biotechnology Information database (handle: asthmagene, submitter: michael kabesch) and have now been included in dbSNP.

‡SNP leads to an amino acid change.

§Data determined on population-based genotyping (n = 1940).

||Data determined in screening population (n = 40).

¶This SNP showed an MAF of 5% in the screening population, but the MAF decreased to 2% in the Dresden population. Therefore the polymorphism was excluded from further analysis because its MAF is less than 3% in the genotyped population.

#Tagging SNPs revealed significant deviations from Hardy-Weinberg equilibrium. Therefore these samples were re-genotyped with RFLP, and previous genotyping results were confirmed. Thus genotyping errors could be excluded in the 2 cases.

phenotype, children without the phenotype were used, irrespective of the other phenotypes. Reference groups for atopic or nonatopic asthma were also nonasthmatic subjects. The dominant model showed the best fit for the *HLX1* data. Results of the dominant model are reported, resulting in *P* values for Pearson χ^2 tests, odds ratios (ORs), and 95% CIs. Logistic regression analysis was used to calculate adjusted ORs, and *P* values of the Wald test are reported in adjusted analyses (see Table E4 in this article's Online Repository at www.jacionline.org). Haplotype frequencies were estimated by using the EM algorithm, and haplotypes with frequencies of greater than 0.03 were analyzed with haplotype trend regressions, in which the estimated probabilities of the haplotypes are modeled in logistic regression as independent variables.²⁰

A Bonferroni correction was used for every phenotype of each tagging SNP to check for multiple testing in the single-SNP analyses of the pooled population (n = 3099). Haplotype analyses were corrected for the number of common haplotypes. Additionally, a homogeneity analyses was performed in 2 different populations (Dresden, n = 1940; Munich, n = 1159) to validate the effects observed for tagging SNPs. All tests were 2-sided, and the differences were considered significant at a *P* value of .05 or less. Calculations were carried out with SAS (9.1.3) and SAS/Genetics software (SAS Institute, Inc, Cary, NC).

Transcription factor binding site analysis was performed with MatInspector²¹ and Alibaba 2.1²² software.

RESULTS

Mutation screening and polymorphism identification in the *HLX1* gene

By using overlapping PCR fragments, the complete length of the *HLX1* gene, including all exons, introns, and flanking regions,

was sequenced in 40 adult volunteers of German origin. Nineteen SNPs with minor allele frequencies (MAFs) of at least 3% were identified in the region of the *HLX1* gene (Table I), whereof 3 (A-1663G, C-796A, and C+3958T) polymorphisms were previously not described and submitted to the public SNP databases (dbSNP). Six polymorphisms were found in the promoter region, 3 in the 5'UTR, 5 in the intronic region, and 1 in the 3'UTR. Four SNPs were located in the coding region, of which 3 resulted in a putative amino acid change in the HLX1 protein.

HLX1 SNP selection for genotyping

The LDs between polymorphisms showing an MAF of at least 3% were calculated to identify tagging SNPs for the genotyping of the *HLX1* gene (Fig 1). Based on the screening population of 80 chromosomes, 5 major LD blocks with r^2 values of 0.8 or greater were observed, whereas 5 SNPs could not be attributed to any LD block in the screening population. These 5 SNPs (C-1407T, C-796A, C-742G, C-559T, and G+4447A) and 1 tagging SNP per LD block (C-1486G, T+346C, G+2256A, T+3183A, and C+3958T) were genotyped in a cross-sectional study population of 1940 children from Dresden, and LD was reassessed (see Fig E1 in this article's Online Repository at www.jacionline.org). In this larger population one of the SNPs (C-796A) was too infrequent to further qualify as an SNP (with an MAF of 2%), whereas all other SNPs could be assigned to one of the 5 previously defined LD blocks (Table I). Tagging

Human chromosome 1q41-42

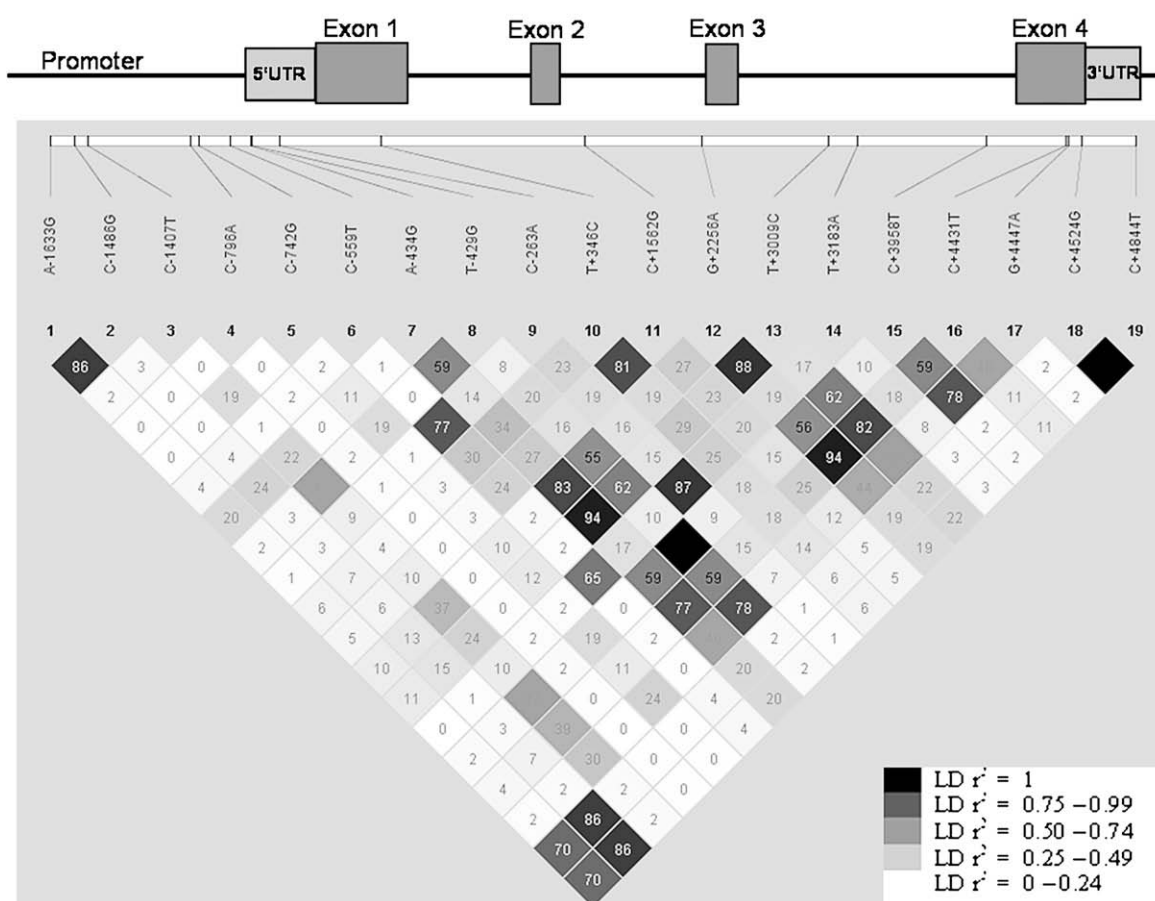


FIG 1. Structure of the *HLX1* gene on chromosome 1q41 and the LD (r^2) plot between the *HLX1* polymorphisms (MAF >3%) identified in the screening population (n = 40). Color code for the LD plot is produced by using Haploview software: white ($r^2 = 0$), shades of gray ($0 < r^2 < 1$), and black ($r^2 = 1$).

SNPs for the 5 LD blocks (all exceeding an MAF of 10%) were then genotyped in the second part of the study population (n = 1159 from Munich).

Association studies with selected *HLX1* SNPs

HLX1-tagging SNPs representing blocks 2 and 3 showed significant associations with asthma in the cross-sectional study population of children from Dresden and Munich (n = 3099, Table II). The polymorphic allele of SNP T+346C, tagging block 3 and leading to a putative amino acid change in exon 1, was protective against the development of asthma (OR, 0.73; 95% CI, 0.56-0.95; $P = .0172$), whereas the minor allele of SNP C-1407T, representing block 2, significantly increased the risk of asthma (OR, 1.44; 95% CI, 1.11-1.86; $P = .0061$). For both SNPs, these effects were slightly stronger for nonatopic asthma. The effect for C-1407T stays also significant after correction for multiple testing in the pooled sample. In addition, the homogeneity analyses demonstrated that the observed effects of both polymorphisms show the same trends in Munich and Dresden (see Table E5 in this article's Online Repository at www.jacionline.org).

Furthermore, the polymorphism at position C-1486G, representing block 1, resulted in a borderline protective effect

concerning nonatopic asthma (OR, 0.64; 95% CI, 0.41-1.00; $P = .0502$). No significant associations with other atopic phenotypes, such as atopic dermatitis, hay fever, or atopy, were observed.

Next, haplotype analyses with the *HLX1* region were conducted (Table III). The haplotype H_b (C-C-C-G-T) showed a borderline significant protective effect against asthma (OR, 0.64; 95% CI, 0.40-1.00; $P = .052$). The haplotype H_c (C-T-T-A-T) significantly increased asthma risk (OR, 1.86; 95% CI, 1.21-2.87; $P = .005$).

HLX1 SNP selection for functional studies

Taken together, association results suggested that *HLX1* LD blocks 2 (C-1407T, C-742G, T-429G, C+3958T, and G+4447A) and 3 (T+346C and C+1562G) might play a role in the pathogenesis of asthma. Functional studies were needed to discriminate those SNPs within the respective LD blocks responsible for the observed effects because the LD was too strong to further dissect the origin of effects by association studies. Within block 2, associated with a significant increase in asthma risk, 2 SNPs (C-1407T and C-742G) were located in the promoter region, 1 in the 5'UTR (T-429G), 1 in intron 3 (C+3958T), and 1 synonymous SNP in exon 4 (G+4447A). Based on the position of these SNPs, we first thought to determine

TABLE II. Associations between *HLX1* tagging SNPs and asthma (divided into atopic and nonatopic asthma), atopic dermatitis, hay fever, and atopy

	Asthma	Atopic asthma	Nonatopic asthma	Atopic dermatitis	Hay fever	Atopy
Nos. (affected/unaffected)	272/2782	124/2782	133/2782	540/2454	280/2752	777/2225
C-1486G (block 1)	0.77 (0.57-1.05)	0.85 (0.55-1.29)	0.64 (0.41-1.00)	0.99 (0.80-1.24)	1.02 (0.76-1.35)	1.00 (0.83-1.21)
<i>P</i> value	.0936	.4364	.0502	.9654	.9201	.9680
C-1407T (block 2)	1.44 (1.11-1.86)	1.39 (0.95-2.02)	1.45 (1.01-2.08)	1.11 (0.91-1.36)	0.95 (0.73-1.24)	1.01 (0.85-1.21)
<i>P</i> value	.0061*	.0870	.0441	.3030	.7050	.8755
T+346C (block 3)	0.73 (0.56-0.95)	0.77 (0.53-1.13)	0.67 (0.46-0.97)	0.93 (0.77-1.13)	1.11 (0.86-1.43)	0.91 (0.77-1.08)
<i>P</i> value	.0172	.1807	.0343	.4841	.4239	.2617
G+2256A (block 4)	1.16 (0.90-1.50)	1.15 (0.79-1.67)	1.10 (0.77-1.56)	1.10 (0.91-1.33)	1.00 (0.78-1.29)	1.08 (0.92-1.28)
<i>P</i> value	.2586	.4716	.6189	.3382	.9825	.3479
T+3183A (block 5)	0.98 (0.76-1.26)	0.96 (0.67-1.38)	1.10 (0.77-1.58)	0.98 (0.81-1.19)	1.04 (0.81-1.34)	1.14 (0.97-1.35)
<i>P</i> value	.8591	.8191	.6061	.8413	.7545	.1213

Associations are given in terms of ORs (95% CIs) for dominant effects in the pooled cross-sectional study population (pooled population, total *n* = 3099). Significant associations are shown in boldface.

*Significant after correction for multiple testing.

TABLE III. Estimated haplotype frequencies in the pooled cross-sectional study population (total *n* = 3099) built of the 5 tagging SNPs (C-1486G, C-1407T, T+346C, G+2256A, and T+3183A) and association tests for individual haplotypes with asthma

	C-1486G	C-1407T	T+346C	G+2256A	T+3183A	Nonasthmatic subjects (n = 2522)	Asthmatic subjects (n = 245)	OR (95% CI) HTR	<i>P</i> value HTR
H _a	C	C	T	G	A	34.30%	34.49%	1.02 (0.70-1.48)	.935
H _b	C	C	C	G	T	24.56%	20.61%	0.64 (0.40-1.00)	.052
H _c	C	T	T	A	T	18.75%	24.08%	1.86 (1.21-2.87)	.005*
H _d	G	C	T	A	T	14.35%	12.65%	0.76 (0.44-1.30)	.315
H _e	C	C	T	G	T	7.26%	7.14%	0.97 (0.48-1.96)	.929
			All rare			0.78%	1.02%		

P values, ORs, and 95% CIs are shown by haplotype trend regression (HTR). Significant associations are shown in boldface.

*Significant after correction for multiple testing.

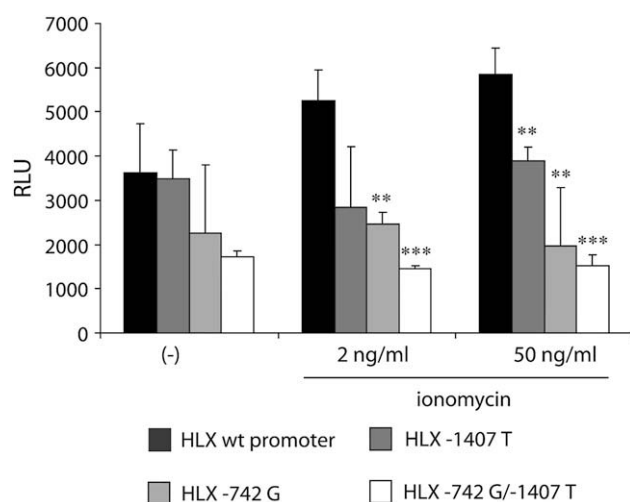
whether the 2 promoter SNPs might lead to a change in *HLX1* gene expression.

Promoter polymorphism-dependent *HLX1* gene expression analysis

Luciferase reporter constructs were generated with 1.8 kb of *HLX1* proximal promoter, carrying either wild-type or polymorphic alleles at positions C-1407T, C-742G, or both. These constructs were transfected into the human T-cell line, and polymorphism-dependent effects on promoter activity were studied after mitogen stimulation (ionomycin) by using luciferase reporter assays. As indicated in Fig 2, the presence of these polymorphic promoter alleles led to a significant decrease in promoter transactivation after stimulation with 50 ng of ionomycin *in vitro*, each by itself (*P* = .003 and .006) and in combination (*P* = .0002). These data suggested that both SNPs in block 2 influence *HLX1* promoter activity independently but with synergistic effects. Thus we then thought to determine the mechanisms by which these changes in promoter activity can occur.

Transcription factor binding analysis of *HLX1* promoter polymorphisms

In silico transcription factor binding analyses with MatInspector and Alibaba Web-based bioinformatic tools were conducted that predicted SNP-dependent alterations of SP transcription factor binding at position -1407. Next, EMSAs were performed for both promoter loci. Radioactively labeled 31mer oligonucleotides,

**FIG 2.** The polymorphisms C-1407T and C-742G reduce *HLX1* promoter activity in Jurkat T cells. Jurkat T cells transiently transfected with 1.8-kb *HLX1* promoter reporter constructs carrying the wild-type (wt) or respective polymorphic sequences (*n* = 3) are shown. Cells were left unstimulated or stimulated with ionomycin and harvested after 18 hours. **P* < .05, ***P* < .01, and ****P* < .001, Student *t* test.

either carrying the wild-type or the polymorphic allele, at the respective positions were used and incubated with nuclear extracts from unstimulated Jurkat T cells (Fig 3). Antibody supershift experiments were performed to specifically identify transcription

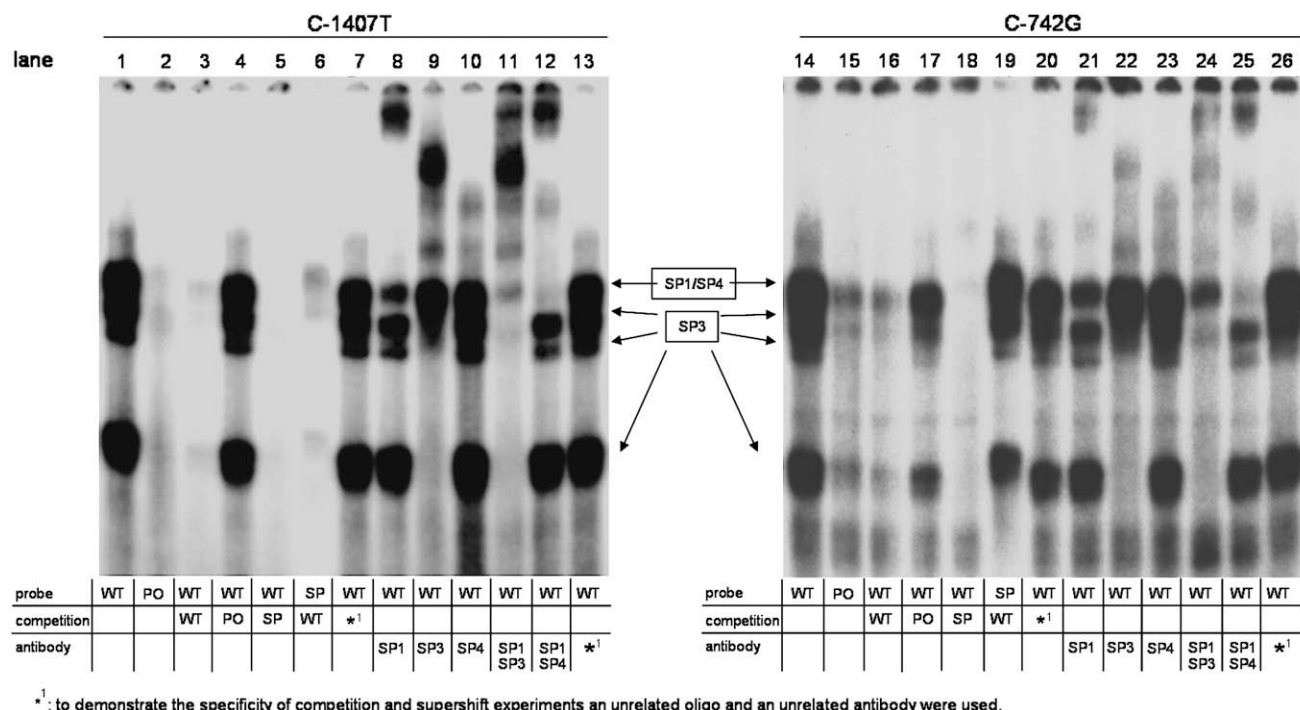


FIG 3. Allele-specific transcription factor binding analyses within the *HLX1* promoter in nuclear extracts from Jurkat T cells. EMSA probes carrying wild-type (WT) or polymorphic (PO) allele for C-1407T (left) and C-742G (right). DNA-protein interaction patterns for wild-type (lanes 1 and 14) and polymorphic alleles (lanes 2 and 15) show the formation of 5 specific complexes in wild-type probes only. All 5 complexes are abrogated by competition with 100-fold molar excess of cold oligos (lane 3 and lane 16) and with SP consensus sites (lanes 5 and 18). Specific antibodies for the SP transcription factor family (SP1, SP3, and SP4) supershift specific complexes (lanes 8-12 and 21-25), indicating the presence of the respective SP proteins in the complexes.

factors within observed DNA protein complexes to confirm the specificity of protein competition experiments. EMSA experiments at both promoter positions (–1407 and –742) revealed strong and significant differences in allele-specific transcription factor binding of SP transcription factors. Although SP transcription factor family members did constitutively bind to the respective wild-type alleles (C-1407 and C-742), this binding was almost completely abolished in the presence of the polymorphic allele (–1407T and –742G). These results suggest that altered SP binding at these promoter positions occurs, which might explain the differences in gene expression observed in luciferase reporter studies.

DISCUSSION

In this study we resequenced the *HLX1* gene locus and identified novel polymorphisms in the gene. Haplotype structure was assessed, and genotyping was performed accordingly, identifying associations between *HLX1* SNPs and asthma in a large study population of 3099 German children. When promoter polymorphisms positively associated with the development of asthma were studied functionally, significant differences in promoter transactivation on ionomycin stimulation were observed, potentially because of significantly reduced binding of SP transcription factors in the presence of polymorphic alleles in the *HLX1* promoter.

Although previous genetic studies investigating the role of transcription factors involved in the T_H1/T_H2 differentiation and

the development of asthma were focused on T-bet (T_H1)⁷⁻¹¹ and GATA3 (T_H2) only,^{23,24} our data suggest that genetic *HLX1* variants might also influence asthma. Until now, no systematic screening for polymorphisms in the *HLX1* gene has been carried out. Thus we resequenced the complete gene locus in 80 chromosomes. It is very unlikely that frequent mutations/polymorphisms in that region were missed by using this comprehensive approach. By using a pragmatic genotyping strategy based on haplotype tagging SNPs, it can be assumed that all relevant genetic information of the locus has been captured by our association analysis.

In these analyses 2 LD blocks genotyped by tagging SNPs C-1407T and T+346C were significantly associated with asthma, nonatopic asthma, or both in the study population. Asthma risk increased in the presence of polymorphisms from LD block 2, represented by tagging SNP C-1407T, and effects were similar for atopic and nonatopic forms of asthma. In contrast, the polymorphic C allele of SNP T+346C representing block 3 decreased asthma risk. This effect was stronger in nonatopic asthma. Interestingly, no significant associations with other atopic diseases or atopic sensitization were found, even though no mutually exclusive reference groups were used in the analysis. Thus it can be speculated that the effects of genetic variants of *HLX1* observed with asthma might not act through intermediate steps of atopic sensitization but rather have a more direct effect on asthma-specific mechanisms independent of atopy. Potentially, it is the absence of the T_H1 signal and not the increase of the T_H2 signal that induces asthma. This phenomenon is not new and has

been observed previously for other asthma genes. For example, IL-13 influences total serum IgE levels but also has IgE-independent effects on asthma development directly related to pulmonary inflammation and airway smooth muscle cells.²⁵ How *HLX1* polymorphisms influence asthma development is not yet clear. To better understand these mechanisms, we started to dissect *HLX1* SNP function in this study.

The 2 tagging SNPs associated with asthma in this study represent 5 (C–1407T) and 2 (T+346C) SNPs in their respective LD blocks. Each of these SNPs can putatively be causal for the signals observed with the respective tagging SNPs. Because LD in block 2 (C–1407T) is extremely high ($r^2 \geq 0.98$), almost every individual is either wild-type or polymorphic at all 5 SNP loci at the same time. Thus dissection of function on a population level is impossible, and other methods had to be used. Of the 5 SNPs in block 2, 2 SNPs were located in the promoter region, 1 in the 5'UTR, 1 in intron 3, and 1 synonymous SNP in exon 4. Of all these SNPs in block 2, the 2 promoter SNPs seemed the most likely candidates to influence *HLX1* function because of their position in a putative regulatory region.

Indeed, both SNPs lead to a decrease in promoter activity, as shown by luciferase gene expression studies. Interestingly, this effect might be due to the loss of transcription factor binding of the same transcription factors in both loci in the presence of the respective polymorphic alleles. –1407T and –742G lead to an almost complete loss of SP transcription factor binding in both regions.

Members of the SP transcription factor family share a highly conserved DNA binding domain, which is able to recognize GC- or GT-rich motifs frequently existing in various promoters.²⁶ Although these factors show a high homology to each other, they exhibit different functional properties.²⁶ SP1 generally induces promoter activity, whereas SP3 is a bifunctional regulator, either activating or suppressing transcription by inhibiting the stimulatory role of SP1.^{27,28} Because SP1 and SP3 compete for the same binding sites, the ratio of SP1 and SP3 is a critical determinant of the influence of SP response elements on transcriptional activity.²⁶ A similar situation was recently described for the polymorphic *CD14* promoter site, a gene that is also involved in IgE regulation and the development of atopy.¹⁷ In that case the polymorphic allele also reduces the binding of transcription factors of the SP family and enhances gene expression, thereby influencing the development of atopy.¹⁷

Although the first functional results evaluating the promoter SNPs in block 2 are promising and suggestive, a number of questions remain. How decreased *HLX1* expression might increase asthma risk is unclear, and a number of intermediate steps need to be studied further in the process from *HLX1* gene expression to disease development. *Ex vivo* studies need to confirm our *in vitro* results. Furthermore, also less likely to be causative for the observed effects, a function of the remaining 3 SNPs in block 2 cannot be excluded yet. Also, in the study reported here, no functional characterizations of SNPs in block 3 have yet been performed. Obviously these polymorphisms must be evaluated in future studies. In particular, polymorphism T+346C, located in exon 1, which leads to an amino acid change from serine to proline, requires further attention. Analyses investigating putative synergistic effects between polymorphisms in *HLX1* and the gene encoding T-bet are currently ongoing.

Taken together, the functional studies thus far performed for 2 promoter polymorphisms associated with an increased asthma

risk at the population level indeed suggest that these polymorphisms alter transcription factor binding sites and expression levels of the *HLX1* gene. Thus *HLX1* might not only be important for T_H1 cell differentiation, but genetic variants in this T-bet-associated gene might also modify the development of asthma.

We thank Tanja Kaacksteen and Ilona Dahmen for expert technical assistance. This work is part of the PhD thesis of Kathrin Suttner and part of the MD thesis of Isabell Ruoss.

Clinical implications: *HLX1* polymorphisms modify childhood asthma risk, stressing the exquisite role of T-cell transcription factors in asthma disease development.

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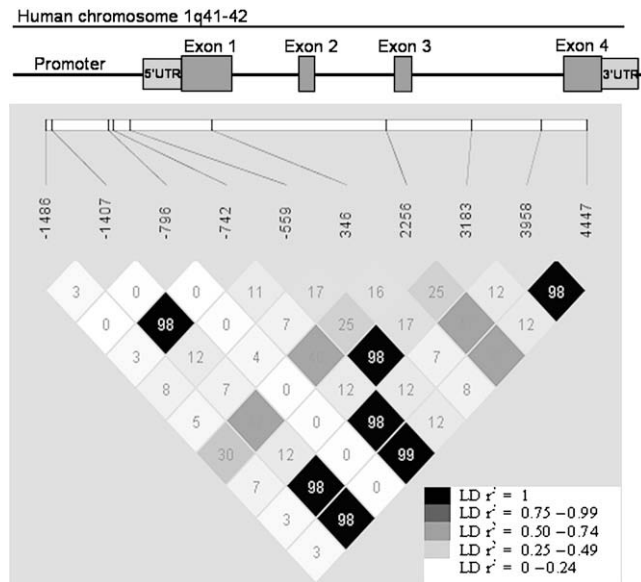


FIG E1. LD (r^2 plot) between *HLX1* single and tagging SNPs (representing blocks 1, 2, 3, 4, and 5) genotyped in the Dresden population ($n = 1940$). Color code for the LD plot is produced by using Haploview: *white* ($r^2 = 0$), shades of *gray* ($0 < r^2 < 1$), and *black* ($r^2 = 1$).

TABLE E1. Descriptive characterization of the International Study of Asthma and Allergy in Childhood phase II study population comparing all genotyped German children included in this analysis (n = 3099)

	No.*	Percentage
Male sex	1561/3099	50.4
Age (y)†	9.6 (0.6)	
Asthma	272/3054	8.9
Atopic asthma‡	124/2906	4.3
Nonatopic asthma §	133/2915	4.6
Hay fever	280/3032	7.3
Atopic dermatitis	540/2994	18.0
Atopy = sensitization	777/3002	25.9
FEV ₁ §	99.8 (10.4)	

*Number affected/number with data available.

†Presented as the mean (SD).

‡For a maximum of 3054 of 3099 children, genotyped information on the respective phenotypes was available. For asthma, 2782 had no asthma reported, 124 were given a diagnosis of atopic asthma (asthma and concurrent positive skin prick test response), 133 were given a diagnosis of nonatopic asthma, and atopy status was not defined for 15 asthmatic subjects.

§FEV₁/mean for n = 1585 for whom this variable was available.

TABLE E2. Description of the primers used for PCR amplification and sequencing of the *HLX1* gene

Fragment	Length	Sense 5'-3'	Antisense 5'-3'
F1	628	TAA AAG AGA GAT GGA GAG CG	CTG CTT TCA ATT CTG TGA CAG
F2	579	CTG CGT TCT AAA GAC CCA AG	CCT TCT CTT GAC CTC ATT CT
F3	599	ACT CAG GGC GGC AAC ATT TG	AGT CCG TTA GTT AGG GGG GG
F3a	516	CAG AAG TTA GAA GAG AGT CAG AGC CC	GAC ACC GCC TCG CTC CC
F4	504	ACT CCC CGC TCC CAT TGG TC	TTG GAG GCG TAG AAG GGA GC
F5	539	CCA CCC ACC CAG TCC GGC TG	CCT CTC AGC GTG TTG CCT T
F6	586	ACC ACA GTG GCT CTG CCC CG	TCC AAG AAC GCC ACA GAC GC
F6a	581	ACA AAC AAA AAA TGA CTC CAG GG	CGG TTA GCA GGG AAG TGA GAT C
F7a	630	GCA AGG GGA AGG GGA GGA AC	ACC CAG ACA TAA ACT CGC CAA GC
F8a	567	GAT CCC AGA AAG TGT GTG CGT G	GCA GAA CGG GAG CCA GAG G
F9	635	GCT GTC ATC ATT CAG GCC CA	AAG ATC TTG CGT TTG CAC CG
F9a	527	CGG TGT ATG TGC GAA GGG C	CCT CAC CCC ATC TCC ACC C
F10	540	AAG GCA GTT CTG GCT CCA GC	GTG TGG CCA CTC CAA GCA GA
F11	594	CCC AGT TGT GAG GAA GTG AA	CCC AAC GCA TTC ACA CAA GT
F12	668	TTA CAC AAC TCT TCT CCC C	CTA AAA TGA CCT GAG TGA C
F12a	467	AGT GGC AGT AGA GGA GAG GGC	ACA CCG AGA TGA GAC CCA GG
F13	641	TGG GCT AGG AGT CCA TAA CT	AGA GAA CGC TCA CTC CCC TC
F13a	519	CTG GTC CTT GGT AGA GTC GCC	GCT GCT GCT ACT AAG ACT GCT GG
F14	618	GAA GCC ATC AGG TGG AGC C	CAC TGA GAC TCC CCT CTG C
F14a	612	CCT CAT TAC CGC CAG CAG	GCC TTA ACA ACC CCC AGC
F15	644	TTG GTA GGG CAG GAG ACG C	ATT GAC CCC GCC TGG CAT C
F16	644	AGC AGG CCA TAG AAA CTG TT	CCT GGA GAG AGG TTT AAC TT

TABLE E3. Description of the primers used for genotyping of the *HLX1* gene in the Dresden and Munich populations

		PCR primers	Extension primer
C-1486G	Second	ACG TTG GAT GTA GAA CGC AGG GTA GTG GTG	TAG TGG TGG TGG AGG TTT T
	First	ACG TTG GAT GGT GGG GAA ATT CAT TCT GAC	
C-1407T	Second	ACG TTG GAT GTT CTA AAG ACC CAA GGC ACG	ACC ACC ACG CAG CTC CTC
	First	ACG TTG GAT GAA TTC TGT GAC AGC TTC CCC	
C-796A	Second	ACG TTG GAT GAA GAG TGA GAT TAC CGT CCC	GTG AGA TTA CCG TCC CTT CCC CA
	First	ACG TTG GAT GTT TCC TGA AGC GCT TGT CAC	
C-742G	Second	ACG TTG GAT GTG ACA AGC GCT TCA GGA AAG	ATG AAA GCT CCT GTG ATC AAC
	First	ACG TTG GAT GTT CTA ACT TCT GCA GCT CCG	
C-559T	Second	ACG TTG GAT GTC ATC CTC GTG ACC AAT GGG	GGA GGG GAA TTA GGA AC
	First	ACG TTG GAT GGC AAA AAC TTT GGC GTG GCC G	
T+346C	Second	ACG TTG GAT GTC CGA AGT CCC GGC TGG CTT	GCG GCT GTC TCC GCT C
	First	ACG TTG GAT GTG TTG TTG TTG CGG GTG ATG	
G+2256A	Second	ACG TTG GAT GGA GCC AGA GGG AAG AAA GAC	AGA AAG ACA AGA GGG C
	First	ACG TTG GAT GAC AGT TTC AAG CCT TAC GGG	
T+3183A	Second	ACG TTG GAT GCA GCA GGT TCT GCC TAT TTG	ATA GGA AAT GTA TTG TGG CTG AA
	First	ACG TTG GAT GGG GAA GCC CAG TTC TTA AAT	
C+3958T	Second	ACG TTG GAT GGA CCC CCA ACA TAC TCT CTC	TTT TTT TAT GCC AGG ACC CCC AT
	First	ACG TTG GAT GCT GGG CTA GGA GTC CAT AAC	
G+4447A	Second	ACG TTG GAT GTC TCA GCC TCG CCT TCA GAA C	GGC TCC TCT CGT CCT G
	First	ACG TTG GAT GAG AAG CCA TCA GGT GGA GCC	

TABLE E4. Comparison of unadjusted and adjusted analyses (adjusted for sex and environmental tobacco smoke exposure)

		Asthma	Atopic asthma	Nonatopic asthma	Atopic dermatitis	Hay fever	Atopy
	Nos. (affected/unaffected)	272/2782	124/2782	133/2782	540/2454	280/2752	777/2225
Unadjusted	C−1486G (block 1)	0.77 (0.57-1.05)	0.85 (0.55-1.29)	0.64 (0.41-1.00)	0.99 (0.80-1.24)	1.02 (0.76-1.35)	1.00 (0.83-1.21)
Adjusted		.0936	.4364	.0502	.9654	.9201	.9680
		0.79 (0.58-1.08)	0.90 (0.59-1.38)	0.64 (0.40-1.01)	1.00 (0.81-1.24)	1.05 (0.79-1.40)	1.05 (0.86-1.27)
		.1421	.6199	.0556	.9981	.7527	.6470
Unadjusted	C−1407T (block 2)	1.44 (1.11-1.86)	1.39 (0.95-2.02)	1.45 (1.01-2.08)	1.11 (0.91-1.36)	0.95 (0.73-1.24)	1.01 (0.85-1.21)
		.0061*	.0870	.0441	.3030	.7050	.8755
Adjusted		1.49 (1.14-1.94)	1.37 (0.93-2.01)	1.57 (1.08-2.27)	1.11 (0.90-1.35)	0.95 (0.72-1.24)	0.75 (0.63-0.90)
		.0033	.1121	.0168	.3277	.6990	.9138
Unadjusted	T+346C (block 3)	0.73 (0.56-0.95)	0.77 (0.53-1.13)	0.67 (0.46-0.97)	0.93 (0.77-1.13)	1.11 (0.86-1.43)	0.91 (0.77-1.08)
		.0172	.1807	.0343	.4841	.4239	.2617
Adjusted		0.73 (0.56-0.96)	0.76 (0.52-1.12)	0.69 (0.47-1.01)	0.93 (0.77-1.13)	1.11 (0.86-1.43)	0.91 (0.77-1.08)
		.0235	.1656	.0552	.4845	.4429	.2767
Unadjusted	G+2256A (block 4)	1.16 (0.90-1.50)	1.15 (0.79-1.67)	1.10 (0.77-1.56)	1.10 (0.91-1.33)	1.00 (0.78-1.29)	1.08 (0.92-1.28)
		.2586	.4716	.6189	.3382	.9825	.3479
Adjusted		1.20 (0.92-1.56)	1.18 (0.81-1.71)	1.14 (0.79-1.64)	1.10 (0.91-1.33)	1.01 (0.78-1.31)	1.11 (0.93-1.31)
		.1761	.4021	.4937	.3333	.9320	.2380
Unadjusted	T+3183A (block 5)	0.98 (0.76-1.26)	0.96 (0.67-1.38)	1.10 (0.77-1.58)	0.98 (0.81-1.19)	1.04 (0.81-1.34)	1.14 (0.97-1.35)
		.8591	.8191	.6061	.8413	.7545	.1213
Adjusted		0.92 (0.71-1.20)	0.94 (0.65-1.35)	1.01 (0.70-1.46)	1.00 (0.82-1.21)	1.02 (0.79-1.32)	1.11 (0.94-1.32)
		.5507	.7237	.9458	.9787	.8731	.2236

Values are presented as ORs (95% CIs) and P values, as shown, and adjusted for sex and environmental tobacco smoke. Current environmental smoke exposure was defined as any current environmental tobacco smoke exposure at the age of 9 to 11 years, according to the information derived from parental questionnaires. Significant associations are shown in boldface.

TABLE E5. Associations between *HLX1*-tagging SNPs and asthma (divided into atopic and non atopic asthma) and atopy in the homogeneity analysis

SNP	Populations	Asthma		Atopic asthma		Nonatopic asthma		Atopy	
	Nos. (affected/ unaffected)	272/2782		124/2782		133/2782		777/2225	
		OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
C-1486G (block 1)	MD	0.77 (0.57-1.05)	.0936	0.85 (0.55-1.29)	.4364	0.64 (0.41-1.00)	.0502	1.00 (0.83-1.21)	.9680
	D	0.99 (0.67-1.48)	.9766	0.95 (0.53-1.71)	.8638	0.95 (0.54-1.69)	.8702	1.08 (0.85-1.36)	.5452
	M	0.54 (0.33-0.86)	.0087	0.70 (0.38-1.29)	.2511	0.36 (0.17-0.76)	.0051	0.90 (0.66-1.23)	.5143
C-1407T (block 2)	MD	1.44 (1.11-1.86)	.0061	1.39 (0.95-2.02)	.0870	1.45 (1.01-2.08)	.0441	1.01 (0.85-1.21)	.8755
	D	1.45 (1.02-2.05)	.0379	1.71 (1.03-2.84)	.0348	1.15 (0.69-1.91)	.5969	1.10 (0.89-1.37)	.3765
	M	1.42 (0.96-2.10)	.0777	1.07 (0.60-1.90)	.8170	1.84 (1.09-3.12)	.0204	0.87 (0.65-1.17)	.3680
T+346C (block 3)	MD	0.73 (0.56-0.95)	.0172	0.77 (0.53-1.13)	.1807	0.67 (0.46-0.97)	.0343	0.91 (0.77-1.08)	.2617
	D	0.62 (0.43-0.89)	.0097	0.72 (0.42-1.21)	.2108	0.50 (0.29-0.86)	.0113	0.87 (0.70-1.07)	.1949
	M	0.91 (0.62-1.35)	.6436	0.89 (0.51-1.54)	.6692	0.95 (0.55-1.62)	.8350	0.97 (0.73-1.29)	.8290
G+2256A (block 4)	MD	1.16 (0.90-1.50)	.2586	1.15 (0.79-1.67)	.4716	1.10 (0.77-1.56)	.6189	1.08 (0.92-1.28)	.3479
	D	1.28 (0.90-1.82)	.1639	1.47 (0.87-2.47)	.1463	1.01 (0.62-1.66)	.9578	1.18 (0.96-1.46)	.1176
	M	1.00 (0.68-1.46)	.9987	0.85 (0.49-1.45)	.5418	1.14 (0.68-1.92)	.6131	0.94 (0.71-1.24)	.6388
T+3183A (block 5)	MD	0.98 (0.76-1.26)	.8591	0.96 (0.67-1.38)	.8191	1.10 (0.77-1.58)	.6061	1.14 (0.97-1.35)	.1213
	D	0.89 (0.63-1.25)	.4932	0.67 (0.41-1.12)	.1235	1.35 (0.81-2.26)	.2488	1.02 (0.83-1.26)	.8606
	M	1.12 (0.76-1.64)	.5688	1.44 (0.84-2.49)	.1839	0.92 (0.55-1.53)	.7358	1.41 (1.06-1.88)	.0189

Associations are given in terms of ORs (95% CIs) for dominant effects in the pooled population (MD; total n = 3099) and in the single populations from Dresden (D; n = 1940) and Munich (M; n = 1159). Significant associations are shown in boldface.