

Promoter polymorphism in the 5-lipoxygenase (ALOX5) and 5-lipoxygenase-activating protein (ALOX5AP) genes and asthma susceptibility in a Caucasian population

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Summary

Background 5-Lipoxygenase (5-LO) and 5-lipoxygenase-activating protein (FLAP) are essential for cysteinyl-leukotriene (cys-LT) production, critical mediators in asthma.

Objective We sought to identify novel promoter polymorphisms within the FLAP (ALOX5AP) gene promoter and test the role of these and the previously identified 5-LO (ALOX5) Sp1 promoter polymorphism in asthma susceptibility.

Methods To assess genetic association with asthma phenotypes, we genotyped 341 Caucasian families (containing two asthmatic siblings) and non-asthmatic control subjects ($n = 184$). Genetic association was determined by case-control and transmission disequilibrium test (TDT) analyses. To determine the functional role of polymorphisms on basal transcription, we generated ALOX5AP-promoter-luciferase constructs and transiently transfected human HeLa cells.

Results A novel G/A substitution at –336 bp and a poly(A) repeat ($n = 19$ or 23) at position –169 to –146 bp were identified in the ALOX5AP promoter. Genotyping found the –336 A and poly(A19) alleles at frequencies of $q = 0.06$ and 0.12, respectively. No ALOX5AP allele was associated with asthma or asthma-related phenotypes in case-control or TDT analyses. ALOX5AP-promoter-luciferase analyses did not support a functional role of the –336 or poly(A) polymorphism in determining basal transcription. The ALOX5 Sp1 polymorphism was predominantly homozygous wild-type 5/5 (frequency $q = 0.70$) and heterozygous 4/5 ($q = 0.23$) genotypes and no allele was associated with asthma or asthma-related phenotypes.

Conclusion Taken together, these data do not support a significant role for these polymorphisms in genetic susceptibility to asthma in the Caucasian population.

Keywords ALOX5, ALOX5AP, asthma, polymorphism

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Introduction

Asthma is a complex multifactorial disease, with both strong genetic and environmental components leading to the expression of disease [1]. The cysteinyl-leukotrienes (cys-LTs) are lipid mediators that play a significant role in bronchoconstriction and airway inflammation, and drugs that target these mediators have clinical efficacy in asthma [2, 3]. Cys-LTs are generated by the 5-lipoxygenase (5-LO) pathway. Following Ca^{2+} -dependent cell activation, arachidonic acid released from membrane phospholipids by cytosolic phospholipase (cPLA_2) is donated to 5-LO by 5-lipoxygenase-activating protein (FLAP), an 18 kDa perinuclear protein. 5-LO is a 78 kDa iron-containing enzyme that translocates from the

cytosol to associate with FLAP, and converts arachidonate in two steps into the unstable intermediate LTA_4 . Conjugation of LTA_4 with glutathione by LTC_4 synthase forms LTC_4 , the first member of the family of cys-LTs, which also includes the extracellular metabolites LTD_4 and LTE_4 .

Both 5-LO and FLAP are essential for LT synthesis [4], and there is accumulating evidence that both 5-LO and FLAP are regulated at the transcriptional level. At steady state, mRNA for 5-LO is barely detectable in many cell lines, but mRNA levels are increased following cell activation [5, 6]. Increased expression of both 5-LO and FLAP mRNA in the peripheral blood leucocytes of asthmatic patients compared to normal subjects suggests a role for transcriptional activation in the pathogenesis of asthma [7]. Increased FLAP protein expression has been described in blood eosinophils of mild asthmatics [8]. Differential gene expression studies have also identified the induction of FLAP mRNA as a feature in asthma patients [9].

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The human FLAP gene (ALOX5AP) has been mapped to chromosome 13q12 [10]. The gene spans more than 31 kb and is composed of five small exons and four large introns [11]. The 5' flanking region has been characterized and includes a TATA box and GRE, AP-1 and AP-2 transcription binding sites, with a predicted transcription initiation site at -74 bp relative to the start codon [11].

The 5-LO gene (ALOX5) has been mapped to chromosome 10q11.2 [12], spans approximately 82 kb, and is composed of 14 exons and 13 introns. In the 5' untranslated region, a major transcription initiation site has been identified 65 bp upstream of the initiation codon (ATG), and several transcription factor binding sites including Sp1, Sp3, Egr-1, GATA-2, NK-κB and AP-2 have been predicted [12, 13]. The promoter region contains no TATA or CCAAT sequences, which are typically in close proximity to the transcription initiation sites of housekeeping genes [12, 13]. Following mutational analysis of the ALOX5 gene promoter, polymorphism of the G+C-rich transcription factor binding region at positions -147 to -176 bp was identified [14]. Polymorphism involved the deletion of one, deletion of two or addition of one zinc finger (Sp1/Egr1) binding sites. The mutant alleles ($n < 5$ or > 5) resulted in reduced binding of Sp1/Egr1 to the polymorphic region and reporter gene transcription [14, 15]. Although only a modest change in transcriptional activation was observed, asthma patients homozygous for mutant alleles showed significantly reduced lung function responses to oral treatment with the 5-LO inhibitor ABT-761 [16].

In the current study, we tested the hypothesis that promoter polymorphisms in the ALOX5 and ALOX5AP genes may be susceptibility markers for asthma. Mutation detection analysis of the ALOX5AP core promoter identified two polymorphisms, a G to A substitution at position -336 and a dichotomous A repeat polymorphism at positions -169 to -146, resulting in 19A and 23A alleles. To test for genetic association with asthma-related phenotypes, these polymorphisms and the previously described ALOX5 Sp1 polymorphism were genotyped in 341 Caucasian families selected for two asthmatic siblings, and in a second control cohort of individuals selected for the absence of asthma or family history of asthma. The transmission disequilibrium test (TDT) and case-control analyses were used to test for genetic association. Promoter-reporter technology was used to determine the influence of the novel ALOX5AP promoter polymorphisms on basal transcription.

Materials and methods

Subjects and clinical assessments

Caucasian families ($n = 341$) from the Southampton area were recruited with at least two biological siblings (aged 5–21 years) with a current physician diagnosis of asthma and taking asthma medication on a regular basis. Serum total IgE levels and specific IgE levels for grass, house dust mite, cat, dog, *Alternaria* and tree allergens were determined by RAST. Skin prick testing was also completed for the same common allergens. At least 14 days after any respiratory tract infection, or use of bronchodilator or anti-allergic medication, baseline lung function tests (FEV₁, best of three values within 5%) were performed by Vitalograph® dry wedge bellows spirometer. Bronchial responsiveness was measured as the provocation concentration of inhaled methacholine required to reduce FEV₁ by 20% (PC₂₀ FEV₁). Methacholine dilutions (0–16 mg/mL) were given at 5-min intervals by a DeVilbiss nebulizer with KoKo dosimeter (PDS Instruments Inc., Louisville, CA, USA) (Table 1). Healthy, non-asthmatic controls with no family history of asthma or allergy were recruited from the same Southampton area as the main study (mean age = 42.3 years, SD 10.6, $n = 184$) (Table 1). DNA was extracted from 10 mL whole blood using a Genomic DNA Maxi-Prep kit (QIAGEN, Crawley, UK). Ethical approval was obtained from the Southampton & SouthWest Hampshire Joint Ethics Committee. This population was a component of that described previously [17].

Molecular methods

The simple sequence length polymorphism (SSLP) in the promoter of ALOX5 was genotyped using the ABI377 DNA sequencer in conjunction with Genescan and Genotyper software (Applied Biosystems, Warrington, UK) using standard protocols. An amplicon encompassing the polymorphic region was generated by PCR: 20 ng genomic DNA template, 0.06 U/μl Jumpstart Taq (Sigma-Aldrich, Poole, England), standard buffer, 2.5 mM MgCl₂, 5% DMSO, 0.2 μM dNTPs containing a 3:1 ratio of dGTP to 7-deaza-dGTP and 0.2 μM of each primer (Table 2) in a reaction volume of 10 μL. Thermal cycling included 94 °C for 6 min, then 30 cycles at 94 °C for 15 s, at 62 °C for 23 s, at 72 °C for 30 s and a final 72 °C extension period for 5 min. Depending on the alleles present, PCR fragments of 262, 268, 274 and

Table 1. Phenotypic characteristics of the study cohort

	Pedigrees	Parents	Non-asthma parents	Asthma parents	Sibling 1	Sibling 2	Controls
Age (years)	24.6	40.5	40.7	40.2	13.0	9.9	42.3
Gender (% male)	51.8	49.9	51.0	47.1	56.9	53.6	47.0
Asthma (% doctor)	60.1	27.8	0	100	100	100	0
Eczema (% questionnaire)	45.6	32.7	25.8	50.8	57.8	62.4	0
Hayfever (% questionnaire)	48.9	46.8	38.0	69.8	64.2	47.0	8.7
FEV ₁ (% predicted)	98.05	100.81	103.39	94.12	94.74	95.62	ND
BHR (1/Lslope+30) × 1000	19.03	24.33	26.79	17.22	14.55	12.00	ND
Log IgE (age corrected)	1.25	0.64	0.49	1.01	1.84	1.93	ND
N	1508	681	492	189	341	338	184

BHR, bronchial hyper-responsiveness; ND, not determined.

Table 2. Primers and probes

Application	Forward primer	Reverse primer	Product (bp)
Mutation detection/OLA Genescan (ALOX5AP)	5'-TCCCTCCCCACTGCCATTAT-3'	5'-ACCATTCTGGACCACGCTGAT-3'	840
	5'-TET-CGTGCTCCTCTGCCAAGC-CCTGCTTC-3'	5'-ATTACACAGTGCCTTCTTCTCCT-3'	116/120
		5'-GCTCTGCCTCCAGCTGCACAACCTG-3'	212/216
		5'-CAGTAAGCTTCTCGAGTGCTTCAGGC-TCCCCAGAGAG-3'	254/258
Genescan (ALOX5)	5'-FAM-AGGAACAGACACCTCGCT-GAGGAGAG-3'	5'-ACCATTCTGGACCACGCTGAT-3'	311/315
		5'-GAGCAGCGAGCGCCGGGAGCCTCGGC-3'	262/268/274/280
Reporter construction	5'CAGT(AAGCTT)(GCTAGC)TCCCT-CCCCACTGCCATTAT-3'	5'CAGT(AAGCTT)(CTCGAG)TGCTTCAGGCT-CCCCAGAGAG-3'	797
OLA	<i>Probe 1</i> 5'-FLU-TGGTGCCTGTGCTGGGCTCG-3'	<i>Probe 2</i> 5'-PHOS-TGCTGGTCATGGTCTTGGGT-BIOTIN-3'	
	5'-FLU-TGGTGCCTGTGCTGGGCTCA-3'		

OLA, oligonucleotide ligation assay; FLU, fluorescein; PHOS, 5' phosphate. Restriction sites are shown in parentheses.

280 bp were obtained corresponding to three, four, five and six copies of the Sp1-binding motif.

Using genomic DNA extracted from 30 asthmatic subjects, an 840 bp fragment of the ALOX5AP promoter (corresponding to -768 to +71) was scanned for the presence of polymorphisms by a combination of solid-phase chemical cleavage [18] and direct sequencing (Applied Biosystems). Jumpstart Taq (0.025 U/ μ L) (Sigma-Aldrich) was used for amplification of genomic DNA template (100 ng) in the presence of standard PCR buffer, 2 mM MgCl₂, 0.2 μ M of each primer, 0.2 mM dNTPs (including fluorescent dCTP (R110, Applied Biosystems) and a 1:100 ratio of unlabelled dCTP for chemical cleavage, to give a final reaction volume of 100 μ L. Thermal cycling included a single soak at 96 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 45 s and a 72 °C soak for 7 min. A PTC-225 DNA Engine Tetrad was used (MJ Research Inc., Waltham, MA, USA).

The -336 G/A polymorphism was genotyped using oligonucleotide ligation assay (OLA) essentially as described [19]. Briefly, the same PCR amplicon encompassing the -336 region used for mutation detection was generated by PCR. The reaction included 0.025 U/ μ L Taq polymerase (Sigma-Aldrich), standard PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs and 0.2 μ M of each primer in a reaction volume of 10 μ L. Cycling conditions included 96 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 64 °C for 45 s, 72 °C for 30 s and a 72 °C soak for 7 min. OLA probe 1 contained a 5' fluorescein label and was allele specific at the 3' terminal. Probe 2 was labelled with a 5' phosphate and a 3' biotin label (Table 2). ELISA plates coated with streptavidin (Sigma-Aldrich) were used to capture ligated probes, which were detected by an HRP-conjugated anti-FITC rabbit polyclonal antibody (DAKO, Ely, Cambridgeshire, England) followed by tetramethylbenzidine substrate (BD Pharmingen, Oxford, England) [19]. The optical density at 450 nm (OD₄₅₀) was measured using an ELISA plate reader (Titertek Instruments Inc., AL, USA). In each plate, alleles 1 and 2 and PCR (no template) controls were included. Typical positive signals were 10-fold higher than background control levels.

The dichotomous poly(A) ALOX5AP polymorphism at positions -169 to -146 was genotyped as described for the Sp1 SSLP. To enable multiplexing of sample loading on the ABI377, four PCR reactions were used with a common forward primer resulting in a range of PCR product sizes (Table 2). PCR conditions for the 116/120 PCR included Jumpstart Taq (0.025 U/ μ L) and genomic DNA template (40 ng) in the presence of standard PCR buffer, 1.2 mM MgCl₂, 0.2 μ M of each primer and 0.2 mM dNTPs to give a final reaction volume of 10 μ L. Thermal cycling included a single soak at 96 °C for 7 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and finally a 72 °C soak for 7 min. For the 212/216 PCR reaction, the conditions were as above, except that 1.5 mM MgCl₂ and a 58 °C annealing temperature were used. For both the 254/258 and 311/315 PCR reactions, the conditions were as described, except that 1.5 mM MgCl₂ and an annealing temperature of 55 °C were used.

Promoter fragments of the ALOX5AP gene corresponding to -768 to -3, including all reported transcription initiation sites [11], were generated by PCR using primers that engineered *Hind*III/*Nhe*I and *Hind*III/*Xho*I restriction sites at the 5' and 3' of the product, respectively (Table 2). Conditions included 5U AccuTaq (Sigma-Aldrich), standard PCR buffer, 2% DMSO, 1.5 mM MgCl₂, 0.2 mM dNTPs and 280 ng genomic DNA template in 100 μ L heated to 96 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 64 °C for 30 s, 68 °C for 45 s and finally a 68 °C soak for 10 min. DNA previously sequenced was used to generate G-A23, G-A19 and A-A23 haplotypes. PCR products were gel purified using a QIAquick kit (QIAGEN) and blunt cloned into the *Sma*I site of pUC18 using established molecular techniques. The cloned DNA fragments were sequenced to confirm the presence of the appropriate alleles and to verify the fidelity of the PCR reaction. The A-A19 haplotype was engineered by the introduction of the -336 A allele into the G-A19 construct using overlap extension PCR [20]. Constructs were subcloned into the pGL3-basic vector (Promega, Southampton, UK) using *Nhe*I and *Hind*III restriction sites and resequenced. Large-scale plasmid preparations were completed using maxi-preps (QIAGEN).

Cell culture, transfection and dual luciferase assay

HeLa cells (purchased from ATCC, Manassas, USA) were maintained at 37°C in humidified 5% CO₂ in MEM (Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin, 100 mg/mL streptomycin (Sigma-Aldrich) and 50 mg/mL gentamicin (Invitrogen). Cultures were maintained between 10⁵ and 10⁶ cells/mL and passaged every 2–3 days. Trypsin/EDTA (Sigma-Aldrich) was used for harvesting the HeLa monolayer.

Cells were grown to 50–60% confluency, harvested, washed with phosphate-buffered saline (PBS) and resuspended at a density of 10⁷ cells/mL in ice-cold PBS. For each series of transfections, water, pGL3basic (–) negative and pGL3-SV40 positive controls were included. Ten micrograms of pGL3 basic construct and 0.2 µg pRL-SV40 (1 : 50 ratio determined experimentally) were combined with 0.8 mL of cells and mixed by pipetting. Cells were transiently transfected by electroporation using a single pulse of 300 V, 500 µF supplied by a GenePulser (Biorad, Hemel Hempstead, UK). Each transfection was resuspended in 10 mL of medium and grown for 24 h.

Cells were harvested and luciferase activity was determined using the Dual Luciferase AssayTM (DLA) (Promega), as described by the manufacturer. Luciferase activity was measured using a dual injector Lucy 1 Luminometer (LabTech, Andover, MA, USA). For each transfection, the luciferase activity was measured in triplicate and the mean of these values was taken for analysis. Different transfection efficiencies were normalized using cotransfection of pRL-SV40 and *Renilla* luciferase activity. The firefly luciferase activities of cells transfected with the different pALOX5AP-Luc constructs were compared following normalization using the G-A23 wild-type construct and designating this construct as 100% luciferase activity.

Genetic association analysis

Genetic association of the ALOX5AP and ALOX5 polymorphisms with asthma and atopy phenotypes was evaluated by TDT [21] and by case-control analyses. This was implemented using STATA 6.0 (Stata Corporation, TX, USA) and a TDT program written in STATA by David Clayton (MRC Biostatistics Unit, Cambridge, UK). Results showing a significance of 10% or less were repeated on TDT/STDT Program 1.1 (<http://spielman07.med.upenn.edu/TDT.htm>). Only the TDT section of this program was used as the data were presented as trios composed of mother, father and first affected child. Analysis was completed for the phenotypes of a positive case report form (CRF) asthma questionnaire (*Have you ever had asthma? Was this confirmed by a doctor? Have you used any medicines to treat asthma, or any breathing problems, at any time in the last 12 months?*). Analysis was also completed for the phenotypic markers: total IgE, specific IgE, bronchial hyper-responsiveness to methacholine (PC₂₀), mean skin prick test (SPT) (>3 mm) and atopy (defined by positive total IgE and/or specific IgE and/or mean SPT). Total IgE values were dichotomized using age-related cut-off values according to UK NEQAS (United Kingdom National External Quality Assessment Service), and specific IgE ≥ 0.35 IU was classified as positive. Methacholine PC₂₀ was dichotomized using American Thoracic Society criteria as previously described [17, 22], at both the <4 mg/mL and ≤16 mg/mL levels. Both STATA and

TDT/STDT Program 1.1 analyses used the first affected sibling where genotype data were available, and the result was considered significant if $P < 0.05$. Case-control was completed using the χ^2 test to compare genotype frequencies between groups. These analyses for the 5-LO (<5 or >5) and FLAP polymorphisms had a power of 70% to detect a 5% level of significance (for the lowest frequency allele). For the FLAP – 336 polymorphism, we could detect an odds ratio of 2.2, for the FLAP PolyA polymorphism 1.9 and for the 5-LO 1.8.

Data analyses

Differences in the mean normalized luciferase activity for different ALOX5AP constructs were compared by ANOVA and *t*-tests. Figures represent mean values ± SEM. Statistical analyses were completed using SPSS version 10 (SPSS Inc., Chicago, USA), and a *P*-value <0.05 was considered to be significant. ALOX5AP haplotype frequencies were estimated using the 'Estimate Haplotype-frequencies (EH)' program [23].

Results

Polymorphism within the ALOX5AP and ALOX5 promoters

Mutation scanning of ~800 bp of the ALOX5AP promoter region using solid-phase chemical cleavage yielded a digestion pattern for hydroxylamine and osmium tetroxide consistent with the presence of two polymorphisms. Subsequent sequencing to identify the novel polymorphisms identified firstly a G–A substitution at position – 336 (CTC[G/A]TGC) and an SSLP (A) involving two alleles (A23/A19) at positions – 169 to – 146 relative to the initiation codon (ATG). The Sp1 SSLP polymorphism within the promoter of ALOX5 described previously was also identified [14].

Genotyping and allele frequencies

Table 1 summarizes the phenotypic characteristics of the study cohorts. Allele frequencies (*q*) were determined for each polymorphism (Tables 3 and 4). The ALOX5AP – 336 A allele was found at a frequency of $q = 0.06$, and the ALOX5AP poly(A) A19 allele was found at a frequency of $q = 0.12$. The ALOX5 Sp1 genotypes in this Caucasian population were found to be predominantly homozygous WT 5/5 ($q = 0.70$) and heterozygous 4/5 ($q = 0.23$) genotypes. Allele frequencies did not differ significantly from Hardy–Weinberg equilibrium, assessed using chi-squared analysis. The Hardy–Weinberg equilibrium is based on the assumption that gene frequencies and genotype ratios in a randomly breeding population remain constant from generation to generation. Although this rule is not absolute, correlation of genotyping data with the Hardy–Weinberg equilibrium gave confidence in the accuracy of genotyping techniques. Haplotype frequencies for the ALOX5AP polymorphisms were estimated to be G-A23 0.817, G-A19 0.121, A-A23 0.054 and A-A19 0.008 [23].

Genetic association analyses

Case-control analyses using asthmatic parents ($n = 189$) and non-asthmatic adult control ($n = 184$) populations did not

Table 3. ALOX5AP genotype frequencies in different sub-populations

– 336	GG	AC	AA	N	q	H-W χ^2 P-value
Pedigrees	1268	175	1	1444	0.06	—
Parents	583	80	1	664	0.06	0.3
Non-asthmatic parents	420	60	1	481	0.06	0.3
Asthmatic parents	163	20	0	183	0.06	0.4
Asthmatic sibling 1	283	38	0	321	0.06	0.3
Asthmatic sibling 2	267	44	0	311	0.07	0.2
Non-asthmatic Controls	159	24	0	183	0.07	0.3

Poly A	A23	A23/19	A19	N	q	H-W χ^2 P-value
Pedigrees	1121	290	25	1436	0.12	—
Parents	512	139	10	661	0.12	0.9
Non-asthmatic parents	367	103	8	478	0.12	0.6
Asthmatic parents	145	36	2	183	0.11	0.8
Asthmatic sibling 1	247	64	6	317	0.12	0.4
Asthmatic sibling 2	239	65	7	311	0.13	0.3
Non-asthmatic controls	135	41	3	179	0.13	0.9

H-W, Hardy-Weinberg

Table 4. ALOX5 genotype frequencies in different sub-populations

Genotype	3/3	3/4	3/5	4/4	4/5	4/6	5/5	5/6	N
Pedigrees	0.001	0.001	0.006	0.029	0.239	0.002	0.705	0.017	1433
Parents	0.003	0.003	0.006	0.026	0.242	0.003	0.702	0.015	662
Non-asthmatic parents	0.004	0.002	0.004	0.025	0.249	0.004	0.697	0.015	478
Asthmatic parents	0.000	0.005	0.011	0.027	0.223	0.000	0.717	0.016	184
Asthmatic sibling 1	0.000	0.000	0.006	0.035	0.221	0.000	0.726	0.013	317
Asthmatic sibling 2	0.000	0.000	0.007	0.033	0.258	0.003	0.673	0.026	306
Non-asthmatic controls	0.000	0.000	0.000	0.016	0.239	0.005	0.696	0.044	184

Table 5. TDT analyses: ALOX5AP

	– 336 G	Transmitted A	P-value	Poly(A) 23	Transmitted 19	P-value
CRF asthma	39	38	0.909	63	67	0.726
Atopy+CRF	37	33	0.633	64	61	0.789
SPT pos+CRF	39	34	0.558	62	63	0.927
Total IgE+CRF	36	33	0.717	62	62	1.000
Specific IgE+CRF	36	30	0.460	59	57	0.853
PC20 4+CRF	34	34	1.000	53	62	0.401
PC20 16+CRF	30	34	0.617	50	48	0.840

establish the presence of the ALOX5AP – 336 A ($P = 0.5$), ALOX5AP poly(A) A19 ($P = 0.7$) or mutant Sp1 ($n > 5$ or < 5) ALOX5 ($P = 0.3$) alleles as constituting risk factors for the development of asthma.

TDT analysis was completed for the following phenotypes: CRF stringent definition of asthma, atopy plus CRF asthma, skin prick positive (SPT) plus CRF asthma, total IgE plus CRF asthma, specific IgE plus CRF asthma, methacholine PC₂₀ <mg/mL plus CRF asthma, and methacholine PC₂₀ <16mg/mL plus CRF asthma, for both ALOX5AP polymorphisms and the ALOX5 polymorphism (Tables 5 and 6). The data in Table 5 demonstrate that neither of the

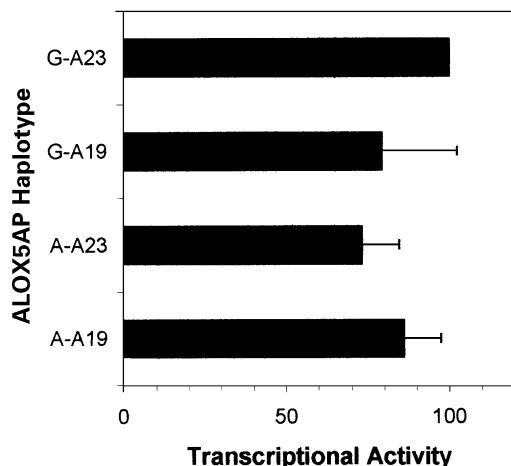
ALOX5AP polymorphisms constituted risk factors for the development of asthma or related phenotypes. Similarly, the ALOX5 Sp1 mutant alleles ($n > 5$ or < 5) were not preferentially transmitted to affected offspring for any phenotype tested (Table 6).

Luciferase reporter assay

To explore the functional significance of the ALOX5AP promoter polymorphisms at the molecular level, promoter fragments containing all four potential ALOX5AP promoter haplotypes were cloned 5' of the luciferase reporter gene

Table 6. TDT analyses: 5-LO

	Allele	Observed allele transmission	Expected allele transmission	χ^2	P-value
CRA asthma	3	2	3	0.667	0.414
	4	78	80	0.100	0.752
	5	90	85	0.588	0.443
	6	4	6	1.333	0.248
Atopy+CRF	3	2	1	2.000	0.157
	4	52	56	0.572	0.450
	5	63	58.5	0.692	0.405
	6	3	4.5	1.000	0.317
SPT Pos+CRF	3	2	3	.	.
	4	75	75.5	0.007	0.935
	5	83	80.5	0.155	0.694
	6	5	6	0.334	0.564
Total IgE+CRF	3	2	3	0.667	0.414
	4	64	65.5	0.069	0.793
	5	76	70.5	0.858	0.354
	6	2	5	3.6	0.058
Specific IgE+CRF	3	3	3	.	.
	4	60	62.5	0.200	0.655
	5	73	68	0.735	0.391
	6	3	5.5	2.273	0.131
PC20 4+CRF	3	2	1	2.000	0.157
	4	52	56	0.571	0.450
	5	63	58.5	0.692	0.405
	6	3	4.5	1.000	0.317
PC20 16+CRF	3	2	2	0.000	1.000
	4	70	70.5	0.007	0.932
	5	77	74	0.243	0.622
	6	2	4.5	2.778	0.096

**Fig. 1.** Promoter-reporter analysis of polymorphic ALOX5AP constructs transiently transfected into HeLa cells ($n = 4$).

within the pGL3 basic reporter vector. Fig. 1 summarizes the data for the transient transfection and luciferase analysis in HeLa cells ($n = 4$). Cells were readily transfected using electroporation, resulting in greater than 200 relative light units (RLU) over background for the pGL3 SV40 promoter positive control vector (background, pGL3 basic vector with no insert). For ALOX5AP constructs, typically values greater

than fourfold over background were observed; these data were normalized relative to the wild-type G-A23 haplotype (designated 100%). The pRL-SV40 vector encoding the *Renilla* luciferase was used to normalize transfection efficiency. No statistical differences in basal ALOX5AP transcriptional activity were identified for the different haplotypes.

Discussion

This study represents the most comprehensive investigation to date of the role of ALOX5AP and ALOX5 gene promoter polymorphisms in determining asthma susceptibility in the Caucasian population. We have identified two novel polymorphisms in the ALOX5AP promoter, a G to A substitution at position -336 , and a poly(A) repeat polymorphism involving two alleles (A23/A19) at positions -169 to -146 relative to the initiation codon. Using genotyping data from 341 asthma-enriched families and 184 non-asthmatic control subjects in conjunction with case-control and TDT analyses, neither the ALOX5AP polymorphism nor the ALOX5 Sp1 polymorphism were identified as risk factors for the development of asthma or asthma-related phenotypes. Functional analysis of the ALOX5AP promoter polymorphism using *in vitro* reporter assay did not suggest that these polymorphisms modulate basal transcription levels.

The -336 G/A polymorphism within the ALOX5AP promoter region has not been described previously. The poly(A) repeat polymorphism (A23/A19) has been described, but the number of A bases was misreported as consisting of 18A and 21A repeats [24]. In this Japanese study of 71 asthmatic and 71 non-asthmatic control subjects, a positive association with asthma was observed for the 21A allele (asthmatics 73.2%; controls 54.9%, $P = 0.035$) [24]. The significance of this case-control analysis is questionable due to the low number of subjects and the potential for error. However, this positive association with asthma in the Japanese population requires further investigation in other cohorts. The absence of a positive association with asthma using our Caucasian subjects may be due to differences in ethnicity and asthma severity between the two study cohorts.

To determine the functional role of ALOX5AP promoter polymorphisms in determining basal transcription, an *in vitro* reporter system was established. Transient transfection of HeLa cells with ALOX5AP-luciferase constructs demonstrated no effect on basal transcription of any permutation of ALOX5AP polymorphisms (Fig. 1). However, the experiments cannot preclude such effects from becoming apparent if cells were appropriately stimulated [25].

Genetic association analyses were also used to explore the significance of the ALOX5 Sp1 polymorphism described by Drazen and colleagues [14] in asthma susceptibility. Using case-control and TDT analyses, no Sp1 variant allele was identified as constituting a genetic risk factor for the development of asthma or asthma-related phenotypes (Tables 4 and 6). To date, this is the first large-scale study designed to test this hypothesis, although previously a small study of 31 asthmatic patients and 25 normal control subjects also did not identify a positive association between any ALOX5 Sp1 allele and asthma [14].

The finding that the ALOX5 polymorphism has a pharmacogenetic effect in asthma patients treated with the 5-LO inhibitor ABT-761 [16] nevertheless extends an understanding of the role of ALOX5 polymorphism in asthma by implicating the ALOX5 gene polymorphism in disease modification, but not disease susceptibility. In 221 asthmatics, those possessing only mutant ALOX5 alleles were relatively resistant to treatment with the LT synthesis inhibitor ABT-761, with mean FEV₁ improving by approximately $18.8 \pm 3.6\%$ ($n = 64$) for WT homozygotes and $23.3 \pm 6\%$ ($n = 40$) for heterozygotes, compared to $-1.2 \pm 2.9\%$ ($n = 10$) in homozygote mutant individuals [16]. The concept of disease-modifying genes in asthma that fail to show genetic association is exemplified by the β_2 -adrenergic receptor polymorphisms [26], which have shown pharmacogenetic effects in asthma patients treated with β_2 -agonists [27] but fail to show genetic association with asthma [28].

In conclusion, asthma is a multifactorial disease with a complex genetic and environmental basis. Multiple studies have reported both positive and negative associations for a wide variety of candidate genes/polymorphisms, including IL-4, IL-13 and the β -chain of the high-affinity IgE receptor (Fc ϵ RI), further confounding conclusions and supporting the need for the publication of negative genetic association data to avoid bias in the literature [1]. The polymorphisms described in the ALOX5AP and ALOX5 gene promoters require analysis in cohorts of greater disease severity and ethnic diversity before final conclusions can be made, but the present study indicates that these polymorphisms do not constitute genetic risk factors for the development of asthma in the Caucasian population. Such evidence does not preclude the possibility that polymorphism in ALOX5 and ALOX5AP may predict those asthmatic individuals in whom LTs make a relatively large pathophysiological contribution, nor that knowledge of a patient's genotype at these loci may provide useful information on the likely clinical response to leukotriene modifier therapy.

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