ORIGINAL ARTICLE

5-Lipoxygenase Pathway Gene Polymorphisms: Lack of Association With Asthma in a Spanish Population

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Abstract

Background: Cysteinyl-leukotrienes are mediators of inflammatory responses in bronchial asthma. We studied the genes encoding the enzymes involved in their synthesis to identify risk factors for asthma. The promoter polymorphisms LTC4S –444 A/C, ALOX5 –176/–147, and ALOX5AP –169/–146 have been reported to be associated with bronchial asthma.
Methods: We analyzed the effects of LTC4S –444 A/C, ALOX5 –176/–147, and ALOX5AP –169/–146 on asthma susceptibility by means of a case-control study with 193 ethnically matched, unrelated individuals. Participants were classified as severe asthmatics, nonsevere asthmatics, and nonasthmatics, using a combination of 2 techniques: polymerase chain reaction-restricted fragment length polymorphism and multiplex capillary electrophoresis.
Results: No association was found between these polymorphisms and asthma, neither individually nor in combination.
Conclusion: Although the studied polymorphisms have been previously reported to constitute risk factors for the disease, we found no association between LTC4S –444 A/C, ALOX5 –176/–147, and ALOX5AP –169/–146 polymorphisms and bronchial asthma.

Key words: Bronchial asthma. 5-Lipoxygenase pathway. Promoter. Polymorphism. Risk.

Resumen

Introducción: los cisteinil-leucotrienos son mediadores de las respuestas inflamatorias que tienen lugar en el asma bronquial. Los genes que codifican las enzimas implicadas en su síntesis han sido estudiadas con el fin de identificar factores de riesgo para esta enfermedad, y los polimorfismos LTC4S –444 A/C, ALOX5 –176/–147 y ALOX5AP –169/–146, localizados en las regiones promotoras de los respectivos genes, han sido asociados al asma bronquial.
Métodos: en este trabajo analizamos los efectos de los polimorfismos LTC4S –444 A/C, ALOX5 –176/–147 y ALOX5AP –169/–146 en la susceptibilidad al asma, mediante un estudio de casos-controles realizado con 193 individuos no relacionados de origen étnico homogéneo, clasificados en pacientes asmáticos graves, pacientes asmáticos no graves y controles no asmáticos. Para las determinaciones genéticas se utilizó una combinación de técnicas de PCR-RFLP y electroforesis capilar múltiple.
Resultados: no se detectó asociación de estos polimorfismos con el asma, ni individualmente ni en conjunto.
Conclusión: Aunque existen trabajos previos que coinciden en considerar los polimorfismos LTC4S –444 A/C, ALOX5 –176/–147 y ALOX5AP –69/–146 como factores de riesgo en el asma bronquial, nuestro estudio no encuentra asociación de los mismos con dicha enfermedad.

Palabras clave: Asma bronquial. Promotor. Polimorfismo. Riesgo. Vía de la 5-lipoxigenasa.

Introduction

Bronchial asthma is a chronic inflammatory disorder of the lower airway affected by both genetic and environmental factors. Cysteinyl-leukotrienes (cys-LTs) are key lipid mediators of the inflammatory responses that characterize bronchial asthma, including bronchoconstriction, tissue edema, eosinophil migration, and stimulation of airway secretions. Increased levels of cys-LTs have been detected in the urine [1,2], exhaled breath condensate [3,4], and sputum [3] of patients with asthma, and in vitro assays have shown greater release of cys-LTs by leukocytes [5,6]. Leukotriene receptor antagonists have clear clinical benefits for some patients [7,8].

Cys-LTs are generated by the 5-lipoxygenase (5-LO) pathway, which is believed to be upregulated based on current experimental evidence and the increased levels of cys-LTs detected in asthmatic patients [9,10]. The promoter regions of the genes involved in this pathway (leukotriene C4 synthase [LTC4S], arachidonate 5-lipoxygenase [ALOX5], and arachidonate 5-lipoxygenase-activating protein [ALOX5AP]) have been screened for regulatory polymorphisms that may affect gene transcription, or that could be associated with the asthmatic phenotype. Several of these polymorphisms have been reported to be associated with bronchial asthma [11-13].

The present study was designed to confirm the association between these polymorphisms, whether individually or in combination, and asthma.

Methods

Patients

A total of 193 unrelated ethnically matched individuals (111 asthmatic and 82 nonasthmatic participants) were recruited at the Allergology Department of our hospital, the reference center for the northern area of Gran Canaria, Spain. Asthmatic patients had a clinical history of wheezing, shortness of breath, chest tightness, and coughing. In all cases, a reversible airway obstruction was demonstrated, fulfilling one of the following criteria: 1) ≥20% difference in peak flow rates for at least 3 days a week, for at least 2 weeks; 2) ≥12% difference or an increase of 200 mL in forced expiratory volume in 1 second (FEV_1) after taking an inhaled $ß_2$-agonist; 3) ≥20% difference in FEV_1 after 1 month of inhaled corticosteroid with or without an associated long-acting $ß_2$-agonist; or 4) ≥20% decrease in FEV_1 after a nonspecific methacholine challenge test. The same physician monitored patients every 3 months for 1 year. At every visit, patients underwent a physical examination and respiratory function tests and answered a questionnaire about asthma and associated diseases. If necessary, treatment was adjusted according to clinical and spirometric criteria and the Global Initiative for Asthma (GINA) guidelines [14]. After the fifth visit, asthma was classified as severe (severe persistent asthma, n=62) or nonsevere (intermittent or mild-moderate persistent asthma, n=49) [14]. We recorded the following data: age at onset, duration of asthma, atopy, total immunoglobulin (Ig) E, specific IgE for *Dermatophagoides pteronyssinus*, baseline FEV_1 and forced vital capacity (FVC), intolerance to nonsteroidal anti-inflammatory drugs (NSAIDs), and treatment. Control participants were recruited from among nonasthmatic patients attending the Allergology Department. They were all healthy, tolerated NSAIDs, and had negative results in skin prick tests (SPTs). The study was approved by the Ethics Committee of Hospital Universitario de Gran Canaria Dr. Negrín, and written informed consent was obtained from all participants.

Measurements

Atopy was defined as 1 or more positive reactions in SPTs, which were carried out in patients and control participants by a standard method using a battery of common aeroallergens (ALK-Abelló, Hørsholm, Denmark): *D pteronyssinus, Dermatophagoides farinae, Tyrophagus putrescentiae, Lepidoglyphus destructor, Alternaria alternata, Olea europaea, Lolium perenne, Cynodon dactylon, Parietaria judaica, Artemisia vulgaris, Plantago lanceolata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum, Blattella germanica*, dog and cat dander, and rabbit epithelium. A wheal diameter at least 3 mm larger than that of the negative control 15 minutes after puncture was considered a positive result. Total and specific IgE to *D pteronyssinus* were measured in an Immulite 2000 analyzer (DPC, Los Angeles, California, USA). The cutoff for positive specific IgE was 0.35 kU_A/L. FEV_1 and FVC were recorded at the fifth visit using a FlowScreen spirometer (Viasys Healthcare, Conshohocken, Pennsylvania, USA). Intolerance to NSAIDs was assessed by controlled oral challenge [15] in patients.

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using a standard method [16]. Polymerase chain reaction (PCR) amplifications were carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, California, USA).

The LTC4S –444 A/C polymorphism was genotyped using a polymerase chain reaction-restriction fragment length polymorphism technique to amplify a 563–base pair (bp) fragment, digestion with the MspI enzyme following the manufacturer's instructions (Bioron GmbH, Ludwigshafen, Germany), and a gel electrophoresis separation of fragments in 3% agarose gels stained with ethidium bromide. Amplifications primers were 5'-TCCATTCTGAAGCCAAAGGC-3' (forward) and 5'-GTCACAGCAGCCAGTAGAGC-3' (reverse). Reactions were performed with 100 ng of genomic DNA, 2 mM dNTPs, 1.5 mM $MgCl_2$, primers (5 pmol each), 10% dimethyl sulfoxide, and 0.5 U of Taq polymerase (Ecogen, Barcelona, Spain) in a total volume of 25 µL. The amplification conditions were as follows: denaturing at 94ºC for 5 minutes, followed by 30 cycles of denaturing at 94ºC for 30 seconds, annealing at 59ºC for 30 seconds, chain elongation at 72ºC for 1 minute, and a final extension step at 72ºC for 10 minutes.

For the ALOX5 –176/–147 and ALOX5AP –169/–146 polymorphisms, the differences between participants were monitored simultaneously based on the variable length of the amplified fragment for each locus using a 15-µL volume reaction containing 50 ng of genomic DNA, 2 mM dNTPs, 1.5 mM $MgCl_2$,

4 pmol of each primer, and 0.5 U of Taq polymerase (Ecogen). The amplification conditions were as follows: denaturing at 94ºC for 5 minutes, followed by 28 cycles of denaturing at 94ºC for 30 seconds, annealing at 58ºC for 40 seconds, elongation at 72ºC for 40 seconds, and a final extension step at 72ºC for 10 minutes. The primer sequences for ALOX5 were 5'-AGGACCAGACACCTCGCTGAGGAGAGAC-3' (forward), 5'- GAGCAGCGAGCGCCGGGAGCCTCGGC-3' (reverse). The primer sequences for ALOX5AP were 5'-GGGAAGTTTCCCATGAACA-3' (forward), 5'- ACCATTCTCGACCACGCTGAT -3' (primer). Forward primers were labelled with the fluorophores 6-FAM and HEX (Applied Biosystems), and product size was determined using ABI PRISM 310 capillary electrophoresis and GeneScan Analysis software v3.1.2 (Applied Biosystems). ALOX5 yielded fragments of 255 bp (12-bp deletion), 261 bp (6-bp deletion), 267 bp (wild-type), and 273 bp (6-bp addition), corresponding to 3, 4, 5, and 6 Sp1/Egr1 binding sites, respectively. For the ALOX5AP polymorphism, fragments of 357 bp and 361 bp were obtained, corresponding to 19 and 23 adenosine repeats, respectively. Several ALOX5 and ALOX5AP samples with homozygous genotypes were further sequenced using the BigDye Terminator v3.1 kit (Applied Biosystems) and the same primers as the PCR in order to verify allele calls.

Statistical Analysis

Deviations from the normal distribution were evaluated by means of the Kolmogorov-Smirnov test, and differences for quantitative variables were assessed by the Mann-Whitney test. The Hardy-Weinberg equilibrium was tested using the χ^2 test, and the effect of polymorphisms on disease susceptibility were determined as odds ratios (OR) and their 95% of confidence intervals (95% CI). All the analyses were performed with SPSS v.13 package (SPSS Inc., Chicago, Illinois, USA), and a P value ≤.05 was considered significant.

Results

Patients and Clinical Phenotype

The phenotypic data of the study participants are shown in Table 1. The proportion of male and female participants was equivalent in the 3 groups, whereas median age differed significantly, that is, it was lower for the nonsevere asthma patients (35 years [range, 18-72 years]) than for the severe ones (44 years [range, 18-79 years]) (P=.002). Females constituted 83.9% and 79.6% of severe and nonsevere asthma patients, respectively. Age at onset, duration of asthma, and intolerance to NSAIDs did not show significant differences between patient groups. The percentage of atopic subjects was significantly higher in the nonsevere asthma group than in the severe one (93.9% vs 69.4%, P=.001). No significant differences were detected for total IgE, but the number of individuals who showed positive *D pteronyssinus* IgE titers was significantly higher for the nonsevere asthma patients (73.5% vs 56.5%, P=.035). As expected, baseline FEV_1 and FVC were significantly lower for the severe asthma patients (70% vs 88% [P<.001] and 70% vs 85% [P<.001], respectively).

When we stratified patients with severe and nonsevere asthma by gender (Table 2, only the data for females are shown), we found that women with severe asthma were older than women with nonsevere asthma (44 years, [range, 20-79 years]) vs 36 years, [range, 18-72 years]; P=.005) and had a lower prevalence of atopy (65.4% vs 94.9%, P=.001). No significant differences were found for male patients with asthma.

5-LO Pathway Polymorphisms and Asthma

Allele frequencies for the 3 polymorphisms are shown in Table 3. All 3 were in Hardy-Weinberg equilibrium in the control population (P=.508, .330, and .144 for LTC4S, ALOX5, and ALOX5AP, respectively).

Table 1. Phenotypic Characteristics of the Study Population[a]

	SA (n=62)	NSA (n=49)	C (n=82)	*P*
Sex, men/women	10/52	10/39	26/56	.076
Age, y	44 (18-79)	35 (18-72)	30 (18-65)	[b]
Age at onset, y	16 (0-53)	13 (0-62)		.169
Duration of asthma, y	23 (3-63)	20 (4-45)		.077
Atopy	43 (69.4)	46 (93.9)		.001
Total IgE, kU_A/L	159 (0-2000)	196 (0-2000)		.785
IgE to *Dermatophagoides pterynossinus* >0.35 kU_A/L	35 (56.5)	36 (73.5)		.035
Baseline FEV_1	70 (32-93)	88 (44-117)		<.001
Baseline FVC	70 (34-89)	85 (54-130)		<.001
Intolerance to NSAIDs	6 (9.7)	7 (14.3)		.556

Abbreviations: C, control group; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; Ig, immunoglobulin; NSA, nonsevere asthma patients; NSAIDs, nonsteroidal anti-inflammatory drugs; SA, severe asthma patients.

[a]Quantitative variables are expressed as median (range), qualitative variables as n (%).

[b]SA vs C, P<.001; NSA vs C, P=.007; SA vs NSA, P=.002.

Table 2. Phenotypic Characteristics of the Female Patients[a]

	SA (n=52)	NSA (n=39)	*P*
Age, y	44.5 (20-79)	36.0 (18-72)	.005
Age at onset, y	16 (0-53)	14 (0-62)	.245
Duration of asthma, y	23 (5-54)	20 (5-45)	.107
Atopy	34 (65.4)	37 (94.9)	.001
Intolerance to NSAIDs	6 (11.5)	5(12.8)	.550

Abbreviations: NSA, nonsevere asthma patients; NSAIDs, nonsteroidal anti-inflammatory drugs; SA, severe asthma patients.
[a] Quantitative variables are expressed as median (range), qualitative variables as n (%).

Table 3. Allele Frequencies

Polymorphism	Allele	SA	NSA	C
LTC4S–444A/C	A	0.75	0.80	0.75
	C	0.25	0.20	0.25
ALOX5–147–176	6[a]	0.02	0.03	0.01
	5	0.78	0.69	0.71
	4	0.18	0.23	0.26
	3	0.02	0.05	0.02
ALOX5AP–169 –146	361[b]	0.84	0.80	0.81
	357	0.16	0.20	0.19

Abbreviations: C, control group; NSA, nonsevere asthma patients; SA, severe asthma patients.
[a] Number of Sp1/Egr1 binding sites.
[b] Base pairs.

Table 4. Risk analysis under a codominant model.

Polymorphism	Genotype	SA+NSA n (%)	C n (%)	OR	95% CI
LTC4S –444 A/C	AA	65 (59.1)	45 (54.9)	1	
	AC	40 (36.4)	33 (40.2)	0.84	0.46-1.52
	CC	5 (4.5)	4 (4.9)	0.86	0.22-3.40
ALOX5 –147/–176	5/5	67 (60.4)	45 (54.9)	1	
	5/m	31 (27.9)	26 (31.7)	0.80	0.42-1.52
	m/m	13 (11.7)	11 (13.4)	0.79	0.33-1.99
ALOX5AP –169/–146	361/361	78 (70.9)	55 (67.9)	1	
	361/357	25 (22.7)	21 (25.9)	0.84	0.43-1.65
	357/357	7 (6.4)	5 (6.2)	0.99	0.30-3-27

Abbreviations: C, control group; CI, confidence interval; m, any non-5 allele; NSA, nonsevere asthma patients; OR, odds ratio; SA, severe asthma patients.

Genotypes were analyzed using codominant, dominant, recessive, and additive models for both groups of patients separately and together (Table 4, only the data for the codominant model and both sets of patients grouped are shown). No association was found for any allele or any tested locus in severe or nonsevere asthma. Similarly, no association was found when considering asthma as a whole.

A test was also performed to detect a possible association between severe asthma or any form of asthma, and the presence of risk alleles for any of the studied polymorphisms (Table 5). Allele C, a number of Sp1/Egr1 binding sites other than 5, and 357-bp alleles were considered as risk alleles for LTC4S –444 A/C, ALOX5 –176/–147, and ALOX5AP –169/–146, respectively. For this analysis, the number of possible risk alleles was categorized as none, 1, 2, and more than 2, in order to strengthen statistical power. Again, we did not find any association between the number of risk alleles and disease.

Table 5. Interaction Effect of LTC4S –444 A/C, ALOX5 – 176/–147, and ALOX5AP –169/–146.

Number of Risk Alleles	SA	NSA	C	Total
0	17 (28.3%)	10 (20.4%)	19 (23.5%)	46 (24.2%)
1	23 (38.3%)	17 (34.7%)	23 (28.4%)	63 (33.2%)
2	13 (21.7%)	15 (30.6%)	29 (35.8%)	57 (30.0%)
>2	7 (11.7%)	7 (14.3%)	10 (12.3%)	24 (12.6%)
Total	60 (100%)	49 (100%)	81 (100%)	190 (100%)

Abbreviations: C, control group; NSA, nonsevere asthma patients; SA, severe asthma patients.

Discussion

The global prevalence of asthma is estimated to increase by 50% every decade [17]. The number of hospital admissions for asthma is also rising, thus reflecting in part an increase in the prevalence of severe asthma [18]. Worldwide, it is estimated that 255 000 people died of asthma in 2005 [19]. Risk factors are both genetic and environmental, and a better understanding of them is important to be able to develop preventive interventions for primary disease. The genes encoding the enzymes of the 5-LO pathway have been widely studied during the last decade. Experimental evidence of the role played by cys-LTs in inflammation, and the observation of higher levels of these lipid mediators in asthmatic patients, has triggered interest in screening the promoter regions of the LTC4S, ALOX5, and ALOX5AP genes in search for variants that might be associated with the disease and account for an effect in this pathway. Since the first report of the association of the LTC4S –444 A/C polymorphism with aspirin-intolerant asthma [11], many studies have tried to verify this result, and have shown the polymorphism to be associated with several asthma phenotypes [20-22], although not all studies have yielded positive results [23-25]. ALOX5 –176/–147 and ALOX5AP –169/–146 have also been analyzed in relation to asthma susceptibility, and again have shown contradictory results [13,26-29].

We performed a case-control study to identify associations between these polymorphisms and the risk for severe persistent asthma, and ultimately for any form of asthma. However, even though we explored 4 different genetic models (dominant, recessive, codominant, and additive) and cumulative effects among loci for association tests, we found no association between the polymorphisms studied and severe asthma, or any form of asthma. In fact, the results for the LTC4S single-nucleotide polymorphism agree with the findings of another study carried out in a Spanish population [25].

Findings on the role of 5-LO pathway polymorphisms as risk factors for asthma are contradictory. Population differences, phenotype definition, population stratification, absence of multiple testing adjustments, interaction effects (eg, there is evidence that a maternal diet supplemented with methyl donors enhances the severity of allergic airway disease in successive generations of mice, and methylation changes in the 5-LO promoter have been demonstrated to be necessary for high 5-LO gene transcription) [30-32], and inappropriate design might account for the lack of conclusive results. As for phenotype definition, the diagnostic procedure used in this study followed the GINA guidelines [14], and the patients were monitored for 1 year before being included in the appropriate group, thus ensuring diagnostic accuracy. However, we studied only a few polymorphisms, and did not analyze all common variations of the genes studied. This could reveal more robust patterns of variations associated with disease [33].

In summary, we performed a case-control study to determine the association between LTC4S –444 A/C, ALOX5 –176/–147, and ALOX5AP –169/–146 promoter polymorphisms and susceptibility to asthma. Although no association was found, interpretations must take into account the limited power reached to detect the modest genetic effects expected.

Acknowledgments

We are grateful to the patients and controls for their collaboration in this study, and to Dr. Carlos Flores for reviewing this manuscript. This study was partially supported by FUNCIS (Fundación Canaria de Investigación y Salud, grant 8/05) and Instituto de Salud Carlos III (Red Respira, Red de Centros C03/11).

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Manuscript received December 17, 2008; accepted for publication March 10, 2009.

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