ORIGINAL

Polymorphisms of the *IL12B*, *IL1B*, and *TNFA* Genes and Susceptibility to Asthma

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Abstract

Background: Asthma is one of the most common chronic inflammatory diseases in developed countries. Susceptibility to asthma is associated with interaction between multiple genes and environmental factors. Several cytokines play a major role in the pathophysiology of the disease.
Objective: We analyzed the distribution of cytokine gene polymorphisms in a group of patients with asthma and a control group in order to determine the effect of these variants, or their combinations, on the development of clinical phenotypes.
Methods: We genotyped 22 single-nucleotide polymorphisms (SNPs) corresponding to 13 cytokine genes (*IFNG*, *IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *TGFB1*, and *TNFA*) in 376 individuals (219 asthmatic patients and 157 controls). Genetic association was evaluated using genotype and allele models for different asthma phenotypes. Gene–gene interactions were explored using multifactor dimensionality reduction.
Results: Genotype AC of *IL12B* -1188 was associated with the presence of asthma. A significant association was detected between 2 SNPs analyzed in *TNFA* (–308 and –238) and atopic asthma and severe-persistent asthma. The *IL1B* TT haplotype (3962T and –511T) was also associated with atopy and moderate-persistent asthma.
Conclusion: Our data show that the presence of SNPs in *IL12B*, *TNFA*, and *IL1B* was significantly associated with asthma, atopy, and severity of asthma. We also highlight the importance of genetic context, haplotype, and gene–gene interaction analysis in genetic association studies.

Key words: Asthma. Atopy. Cytokine. Gene. Polymorphisms.

Resumen

Introducción: El asma es una de las enfermedades inflamatorias crónicas más frecuentes en los países desarrollados. La susceptibilidad al asma viene determinada por la interacción entre múltiples genes y factores ambientales. En la fisiopatología de esta enfermedad las citocinas desempeñan un papel importante.
Objetivo: El objetivo de este estudio fue analizar la distribución de varios polimorfismos en genes de citocinas en un grupo de pacientes con asma y en un grupo control para determinar la influencia que estas variantes, o sus combinaciones génicas, desempeñan en los fenotipos clínicos.
Métodos: Se analizaron 22 SNP correspondientes a 13 genes codificantes de citocinas (*IFNG*, *IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *TGFB1*,and *TNFA*) en 376 individuos, 219 sujetos asmáticos y 157 controles. Las asociaciones genéticas fueron evaluadas empleando modelos genotípicos y alélicos para los distintos fenotipos de asma. Las interacciones gen-gen se analizaron mediante la plataforma *Multifactor Dimensionality Reduction Platform* (MDR).
Resultados: El genotipo AC de *IL12B -1188* se asoció con la presencia de asma. Dos SNP analizados en el gen *TNFA* (*TNFA-308*, and *TNFA-238*) mostraron una asociación significativa con el asma atópica y con la presencia de asma persistente grave. El haplotipo TT de *IL1B* (3962T y -511T) también se asoció con la presencia de atopia y con asma persistente moderada.
Conclusiones: Nuestros datos muestran que la presencia de estos SNP en los genes *IL12B, TNFA* y *IL1B* se asocian significativamente con el asma, la atopia y con la gravedad del asma. También recalcamos la importancia del contexto génico, haplotipos e interacciones gen-gen en los estudios de asociación génica.

Palabras clave: Asma. Atopia. Citocina. Gen. Polimorfismo.

Introduction

Asthma is a chronic inflammatory disease characterized by recurrent respiratory symptoms, reversible variable airway obstruction, airway inflammation, and airway hyperreactivity [1]. It is one of the most common chronic diseases in developed countries, and estimations suggest that 300 million individuals are affected worldwide [2]. The phenotypic heterogeneity of asthma is well documented, and a number of clinical properties such as atopic phenotype or asthma severity have been applied to describe subtypes of asthma and even to manage clinical symptoms [3].

Susceptibility to asthma is related to the interaction between multiple genes and environmental factors. Whereas environmental factors are known to trigger or modulate asthma responses [4], the genetic components that underlie asthma susceptibility are not completely understood. Analysis of single-nucleotide polymorphisms (SNPs) has been widely used for the study of complex genetic disorders. The identification of variations in specific genes involved in the expression of asthma phenotypes could lead to a better understanding of the underlying pathways or even facilitate management tailored to the patient's genotype.

Several studies have reported an association between asthma or atopy and genes coding for molecules involved in various pathways [5]. The study of cytokine genes is particularly important owing to the significant role of cytokines in pathophysiology. In the last decade, many authors have studied cytokine gene SNPs in different populations [6-12]. However, associations vary widely between different ethnic populations. In this sense, characterization of phenotypes following appropriate clinical criteria is a key component of genetic association studies. Other aspects underlying the discrepancies observed in these studies include quality control measures, specifically in laboratory procedures and statistical analysis. Furthermore, given the complex nature of asthma, inconsistencies also arise because of individual approaches to the analysis of polymorphisms, since phenotypes are better explained by analyzing the genetics of asthma in terms of haplotype combinations and gene–gene interactions. The aim of this study was to analyze 22 SNPs of cytokine genes in patients with asthma and to determine the effects of these variants, or their genetic combinations, on asthma.

Material and Methods

Participants

The population of this case-control study comprised 376 individuals (219 asthmatic patients and 157 controls) from the Outpatient Allergy Department of the University Hospital of Salamanca, Salamanca, Spain. The study was performed following the recommendations of the Ethics Committee of the University Hospital of Salamanca. All individuals gave their written informed consent. The inclusion criteria for controls were as follows: no symptoms or history of asthma or other pulmonary diseases; no symptoms or history of atopy; negative skin prick test results with a battery of common aeroallergens (wheal with a diameter <1 mm greater than that of the saline control); and absence of first-degree relatives with a history of asthma or atopy. Asthmatic patients were recruited if they had physician-diagnosed asthma and fulfilled the following criteria: at least 2 symptoms consistent with asthma; a positive methacholine provocation test or bronchodilator test result; and absence of other pulmonary disorders. Severity of asthma was classified following the Global Initiative for Asthma guidelines [13].

Skin Tests and Total Serum Immunoglobulin E Determinations

Skin prick tests were performed according to the recommendations of the European Academy of Allergy and Clinical Immunology [14] with a battery of common aeroallergens that included *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, *Acarus siro*, *Euroglyphus maynei*, *Cynodon dactylon*, *Phleum pratense*, *Cupressus arizonica*, *Platanus acerifolia*, *Olea europaea*, *Quercus ilex*, *Parietaria judaica*, *Artemisia vulgaris*, *Plantago lanceolata*, *Alternaria alternata*, *Cladosporium herbarum*, *Penicillium notatum*, *Aspergillus fumigatus*, *Blattella germanica*, and epithelia of dog, cat, hamster, horse, and rabbit (ALK-Abelló). Saline was used as negative control and histamine (10 mg/mL) as a positive control. Skin tests were considered positive if at least 1 allergen elicited a wheal reaction >3 mm in diameter after subtraction of the negative control. Patients were considered atopic if they had at least 1 positive skin test result. Total serum immunoglobulin (Ig) E was measured using a fluoroenzyme immunoassay (ImmunoCAP, Thermo Fisher Scientific Inc).

Molecular Analysis

Genomic DNA was isolated from peripheral blood white cells using the MagNA Pure Compact Instrument (Roche Applied Science, Mannheim, Germany). A selection of 22 SNPs in 13 genes *(IFNG*, *IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *TGFB1*, and *TNFA*) were genotyped using the Invitrogen Cytokine Genotyping Kit (Heidelberg University). This method is based on the polymerase chain reaction with sequence-specific primers (PCR-SSP), which uses identical amplification and detection conditions, thus enabling rapid and cost-effective analysis of polymorphisms and haplotypes. Amplification was carried out using a 96-well thermal cycler (TProfessional Thermocycler, Biometra) under the following conditions: initial denaturation, at 94ºC for 4 minutes followed by 10 cycles of denaturation at 94ºC for 30 seconds and primer annealing and extension at 65ºC for 1 minute; 20 cycles of denaturation at 94ºC for 30 seconds, primer annealing at 65ºC for 50 seconds and extension at 72ºC for 1 minute. After cycling was completed, the PCR products were loaded into a 2% agarose gel for electrophoresis, and ethidium bromide–stained gel was photographed and interpreted using a worksheet for specific amplification patterns. All laboratory procedures were performed following the European Management Quality Network best practice guidelines.

Statistical Analysis

The allele and genotype frequencies in asthma patients were compared with those of the control population. All groups were tested for Hardy-Weinberg equilibrium using the χ^2 test. The dichotomous variables were analyzed using the χ^2 test, Fisher exact test, odds ratio test, and Monte Carlo simulation (after 10^4 interaction). IgE levels were transformed to $\log_{10}$ values to obtain a normal distribution and compared using analysis of variance. Logistic regression was applied to model the effects of multiple covariates. In the multivariate analysis, sex and age were included as potential covariates. Case-control studies were also undertaken using haplotype combinations of polymorphisms. Haplotype combinations obtained by direct genotyping were compared to those calculated using SNP-Analyzer (ISTECH INC.) based on classical algorithms (expectation-maximization, Clark, and pseudo-Gibbs sampling). Association between genetic variants was estimated using the SHEsis software platform [15] and SPSS version 17.0 (SPSS Inc). All haplotypes with a frequency higher than 1% in either patients or controls were included; this analysis was performed using a normal χ^2 test, odds ratio test, and Monte Carlo simulation. The multifactor dimensionality reduction (MDR) platform (http://www.multifactordimensionalityreduction.org/) based on 1000 permutations was used to explore gene–gene interactions. MDR is a data mining approach for detecting and characterizing combinations of attributes or independent variables that interact to influence a dependent or class variable. Statistical power was calculated at the beginning of the study to determine sample size and at the end of the study to confirm it. False-positive report probability (FPRP) was analyzed to evaluate the effect of multiple combinations.

Results

The clinical characteristics of the asthmatic patients are summarized in Table 1. Total IgE levels were higher in patients than in controls. Since the controls were older, we were able to confirm the absence of asthma and allergy for a longer period.

Allele, Genotype, Haplotype, and Diplotype Analysis

The characteristics of the 22 SNPs analyzed in this study are described in Table 2. The comparative analysis of the allelic and genotypic frequencies between controls and patients with asthma is shown in Table 3. Several positive associations were found, but only those with a statistical power greater than 80% and an FPRP under 10% are provided.

The analysis of *IL12B* –1188 (rs3212227) revealed a strong association with atopic asthma, especially for the AC genotype in asthma patients sensitized to house dust mites (OR, 4.62; 95%CI, 1.83-11.66; *P*=.001).

Allele A of *TNFA* –308 (rs1800629) was associated with atopic asthma, especially in patients with positive skin prick test results to house dust mites and grasses (OR, 3.11; 95%CI, 1.48-6.51; *P*=.001). This SNP was also strongly associated with the severity of asthma. Thus, the AG genotype was more frequent in patients with severe persistent asthma than in controls (OR, 10.55; 95%CI, 3.08-36.20; *P*=.001). In addition, the AG haplotype (combination of allele A for *TNFA* –308 and allele G for *TNFA* –238) was more common in patients with atopic asthma induced by house dust mite and grass (OR, 3.09; 95%CI, 1.48-6.47; *P*=.002) and in patients with severe asthma (OR, 2.25; 95%CI,1.21-4.20; *P*=.008) than in controls (Table 4). Although the statistical power of the haplotype association did not reach 80% (73% [FPRP, 15%]), the diplotype analysis revealed an association between the diplotype AG GG (genotype AG for –308 and genotype GG for –238) and severity of asthma (OR, 2.81; 95%CI, 1.36-5.82; *P*=.005), with a statistical power of 96% (FPRP, 3.4%).

As for *IL1B*, haplotype analysis showed that the TT haplotype (T at position 3962 and T at –511) was significantly associated with severity of asthma and was more common in patients with moderate-persistent asthma (OR, 13.92; 95%CI, 2.99-64.87; *P*<.001) (Table 4). The TT haplotype was also associated with sensitization to molds (OR, 14.70; 95%CI, 2.60-83.09; *P*<.001).

A concordance of >98.8% was detected in the comparison of calculated and genotyped haplotypes. The analysis of *TNFA* –308/–238 showed that the Clark algorithm fits slightly better (99.99%) than the expectation-maximization and Gibbs sampling algorithms (99.8%).

Table 1. Characteristics of the Study Population

	Patients	Controls
Number of subjects	219	157
Age, y, mean (SD)	31 (26)	50 (33)
Women, men	132, 87	99, 58
Log_{10} IgE, mean (SD)	2.20 (0.56)	1.59 (0.57)[a]
Atopic asthma	172 (78.5%)	–
Nonatopic asthma	47 (21.5%)	–
Aeroallergen sensitization		
House dust mites, %	48.8	–
Grasses, %	17	–
Molds, %	8.7	–
Epithelia, %	23.3	–
House dust mites + grasses, %	25.6	–
Polysensitization, %	43	–
Family history of asthma, No.	88	–
Family history of atopy, No.	92	–
Severity of asthma		
Intermittent asthma, %	31.9	–
Mild-persistent asthma, %	22.4	–
Moderate-persistent asthma, %	33.8	–
Severe-persistent asthma, %	11.9	–
Nasal polyposis, No.	29	–
NSAID hypersensitivity, No.	30	–
ASA triad, No.	14	–

Abbreviations: Ig, immunoglobulin; NSAID, nonsteroidal anti-inflammatory drug; ASA triad, aspirin triad.
[a]*P* value for the comparison of $\log_{10}$IgE levels between patients and controls, <.0001 (analysis of variance).

Table 2. Description of the 22 Single-Nucleotide Polymorphisms Analyzed in This Study

Gene	NCBI Identification	Name (Heidelberg system)
IL1A	rs 1800587	*IL-1A -889 C>T*
IL1B	rs 16944 rs 1143634	*IL-1B -511 C>T* *IL-1B 3954 C>T (IL-1B 3962)*
IL1R1	rs 2234650	*IL-1R1 pstI A C>T (IL-1R1 pstI 1970)*
IL1RN	rs 315952	*IL-1RN pos 30,735 T>C IL-1RN mspaI*
IL2	rs 2069762 rs 2069763	*IL-2 -714 T>G(IL-2 -330)* *IL-2 114 G>T (IL-2 166)*
IL4	rs 22432484 rs 2243250 rs 2070874	*IL-4 -1098 T>G* *IL-4 -589 C>T (IL-4 -590)* *IL-4 -33 C>T*
IL4R	rs 1801275	*IL-4R Gln221Arg A>G(IL-4R 1092)*
IL6	rs 1800795 rs 1800797	*IL-6 -174 G>C* *IL-6 -597 G>A (IL-6 nt565)*
IL10	rs 1800872 *rs 1800871* rs 1800896	*IL-10-592 C>A* *IL-10 -819 C>T* *IL-10 -1082 A>G*
IL12B	rs 3212227	*IL-12B pos 1188 A>C (IL-12B -1188)*
IFNG	rs 2430561	*IFN-G 874 A>T*
TGFB1	rs 1982073 rs 1800471	*TGFB-1 869 T>C (TGFB-1 codon 10)* *TGFB-1 915 G>C (TGFB-1 codon 25)*
TNFA	rs 361525 rs 1800629	*TNF-A -238 G>A* *TNF-A -308 G>A*

Abbreviations: NCBI, National Center for Biotechnology Information.

Table 3. Genotypic and Allelic Frequencies of *IL12B* and *TNFA* Single-Nucleotide Polymorphisms

		Genotype				Allele		
IL12B-1188	No.	AA	AC	CC	*P* Value (Fisher)	A	C	*P* Value (Fisher)
Control	139	.705	.252	.043		.831	.169	
Asthma	195	.559	.390	.051	.020	.754	.246	.017
Atopic asthma	155	.561	.381	.058	.038	.752	.248	.018
House dust mites	79	.506	.430	.063	.001[a]	.722	.278	.006
House dust mites/grass	39	.513	.462	.026	.011[a]	.744	.256	.080
TNFA –308	No.	GG	GA	AA	*P* Value	G	A	*P* Value
Control	153	.889	.098	.013		.938	.062	
Asthma	215	.795	.186	.019	.056	.888	.112	.021
Atopic asthma	168	.804	.173	.024	.109	.890	.110	.031
House dust mites/grass	41	.732	.195	.073	.018	.829	.171	.001[a]
Polysensitized	71	.761	.183	.056	.028	.852	.148	.003[a]
Severe-persistent asthma	26	.692	.308	.000	.0001[a]	.846	.154	.021

[a] Results with a statistical power >80% and false positive report probability <10%.

Table 4. Haplotype Analysis of the *IL1B* and *TNFA* Genes

	P Value (Fisher)	OR (95%CI)	Power	FPRP
TNFA –308/–238 AG				
Grass-sensitized	.003	2.61 (1.35-5.02)	81%	7.1%
Grass and house dust mite–sensitized	.002	3.09 (1.48-6.47)	83%	4.8%
Severe asthma	.008	2.25 (1.21-4.20)	73.1%	15.8%
IL1B 3962/–511 TT				
Molds	<.001	14.70 (2.60-83.09)	80%	4.1%
Mild-persistent	<.001	8.53 (1.93-37.82)	81.8%	7.9%
Moderate-persistent	<.001	13.92 (2.99-64.87)	88.3%	1.4%

Abbreviation: FPRP, false positive report probability.

Gene–Gene Interactions

Analysis of gene–gene interaction revealed several combinations associated with atopic and nonatopic asthma. Given the complexity of the statistical analysis, only combinations with *P*<.005 (Fisher) were taken into account. Combinations associated with atopic asthma are shown in Figure 1. Several combinations were detected only in atopic patients. The most significant combination found was *IL12B* –1188/*TGFB1* c10/*IL6* nt565/*IL10* –819 (Figure 1), with a cross-validation consistency (CVC) of 10 and a testing balanced accuracy (TBA) of 33.45% after 1000 permutations (*P*=.001). For this genetic combination, we detected 4 specific sequences (represented as dark gray boxes in Figure 1), which were found only in the group of patients with atopic asthma (AA for *IL12B* –1188, TT for *TGFB1* c10, GG for *IL6* nt565, and TT for *IL10* –819).

The analysis of nonatopic patients showed that the combination TC AA of *TGFB1*c10(TC)/*IL6* nt565(AA) was found only in controls (CVC, 8; TBA, 27.72% after 1000 permutations; *P*=.002) (Figure 2).

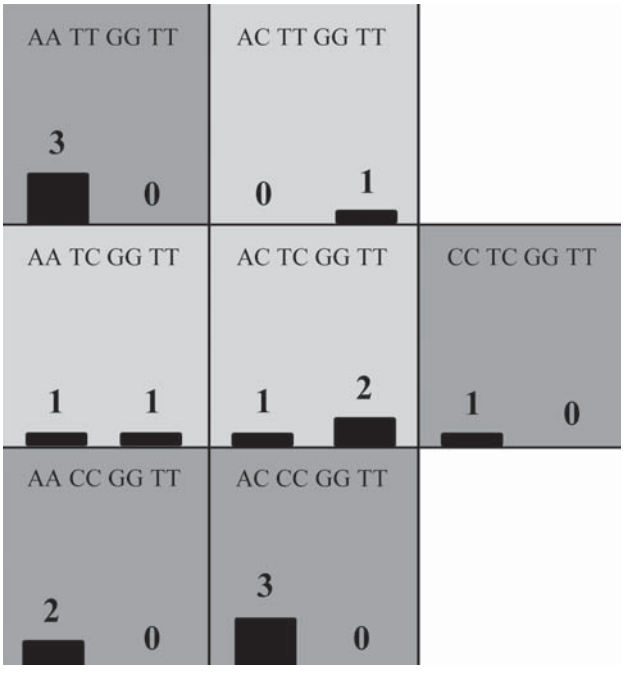

Figure 1. Analysis of *IL12B* –1188/*TGFB* 1c10/*IL6* nt565/*IL10* –819 gene–gene interactions. Each square represents a genetic combination of the 4 SNPs. Combinations with darker shading showed the greatest association with disease. For each combination (each square) the first number (first column) refers to the number of individuals with this combination in the control group, whereas the second number (second column) refers to the number of patients with this combination. It is worth mentioning the involvement of *IL12B* in the association.

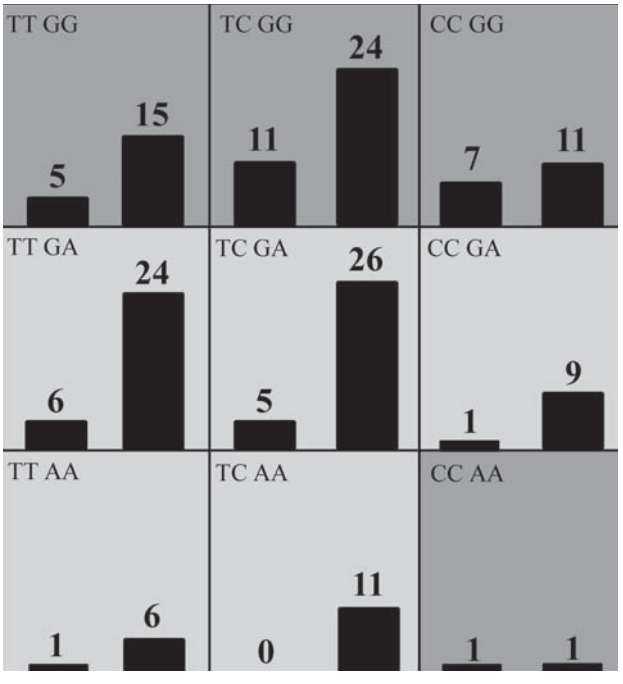

Figure 2. Analysis of *TGFB* c10/*IL6* nt565 gene–gene interactions. Combination TC AA was found only in control individuals by multifactor dimensionality reduction analysis. Each square represents a genetic combination of the 2 SNPs. Combinations with darker shading showed the greatest association with disease. For each combination (each square) the first number (first column) refers to the number of individuals with this combination in the control group, whereas the second number (second column) refers to the number of patients with this combination.

Discussion

In this association study, we analyzed 22 SNPs located in 13 different genes in 376 individuals. The sample size was selected based exclusively on statistical power. We found a strong association between *IL12B* –1188 and atopic asthma, with a >4-fold increased risk for the AC genotype. The cytokine IL12 plays a key role in the immune response, especially in the balance between type 1 and 2 helper T (T_H) cell responses [16]. Previous studies highlighted the role of the *IL12B* –1188 promoter SNP rs3212227 in the expression of the gene [9], although this hypothesis is open to debate [17,18]. Our results reinforce those reported by Morahan et al [9], who suggested that this SNP could lead to a reduction in the expression of IL12 with the consequent activation of the T_H2 response, which could lead to sensitization to several common aeroallergens.

We found that the presence of allele A at position –308 of the *TNFA* gene was significantly associated with atopic asthma and severity of asthma. This association was also detected in the haplotype and diplotype analyses. Tumor necrosis factor (TNF) α is a pleiotropic cytokine involved in many immunological processes. It acts as a proinflammatory cytokine in the airways of asthmatic patients [19,20] and has been studied in allergic sensitization [21-24]. An increase in TNF-α levels has been detected in patients with asthma, particularly in individuals with severe asthma [18]. This finding has been used in the treatment of severe asthma (TNF-α inhibitors), with varying results [25,26]. Our findings reinforce the important role of TNF-α in the clinical management of asthma [27]. The polymorphism *TNFA* –308 has been proposed as one of the genetic factors involved in the variability observed in clinical practice and should be considered when antagonists are proposed as treatment.

We studied 2 SNPs located in the *IL1B* gene (rs16944 and rs1143634) and detected an association between the TT haplotype and severity of asthma. IL-1 is a proinflammatory cytokine with an important role in the immediate immune response. The results detected support the previously reported association between *IL1* cluster polymorphisms in an Iranian asthmatic population [28]. However, the authors did not describe the phenotypic characteristics of asthma in these patients. Several studies have identified an association between SNPs in *IL1B* and nasal polyposis in asthmatic patients [29] and nonasthmatic individuals [30]. SNPs in *IL1B* could deregulate expression of the *IL1* gene.

As for gene–gene interactions, we applied a statistical approximation to perform a combined multifactorial analysis of the SNPs studied in this work. Using the MDR platform, a data mining approach for detecting and characterizing combinations of attributes that interact to influence a phenotype, we simultaneously analyzed the 22 SNPs to find the most significant gene–gene interactions associated with asthma [31-34]. The combined analysis of all polymorphisms provides very interesting data; most of the polymorphisms involved in MDR combinations were not associated with the disease in the single site analysis. In addition, several combinations were detected only in the atopic patients, whereas others appeared only in the control group.

Again the SNPs in *IL12B* were involved in the association with atopic asthma combined with SNPs that were not individually identified. However, the main problem with this approach is that as the number of simultaneously analyzed SNPs increases, the number of patients in each group decreases dramatically, thus hampering detection of predictive combination markers.

Association studies are problematic because of the difficulty in replicating findings in different populations, thus illustrating the importance of performing studies using phenotypic classifications. Consequently, the selection criteria for controls in the present study were very restrictive, and the asthma severity was carefully classified according to appropriate criteria. The statistical analysis also played a key role. We used logistic regression to model the effects of multiple covariates, and sample size was selected strictly based on the analysis of the statistical power. We performed 2 different sample size analyses to confirm statistical power, one at the beginning of the study and the other during the genotyping process. In addition, the effect of multiple comparisons was taken into account in order to avoid type I errors. To control for this effect, we analyzed FPRP and used a more restrictive *P* value (<.005) in the MDR analysis.

In conclusion, our exhaustive analysis of variants in cytokine genes revealed an association between susceptibility to asthma and polymorphisms in *IL12B*, *IL1B*, and *TNFA*. Our analysis of haplotypes, diplotypes, and gene–gene interactions using the MDR platform provides important additional information on more specific asthma-related combinations. Further investigation could reveal how the variants studied regulate cytokines in asthma and their possible role as a therapeutic target.

Funding

This study was funded by the Spanish Fondo de Investigación Sanitaria (grants PS09/02068 and PI10/01706, cofunded by FEDER), the Junta de Castilla y León (grants GRS224/A/08, SAN673/SA03/08, GR255, and HUS01A08), and the Foundation of the Spanish Society of Allergology and Clinical Immunology.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Previous Presentation

XXVI Congress of the Spanish Association of Allergology and Immunology (SEAIC), Bilbao, Spain 2008.

XXVIII Congress of the European Academy of Allergy and Clinical Immunology, Warsaw, Poland 2009.

Annual Meeting of the Aragon - Allergology Association, Huesca, Spain, 2010.

American Academy of Allergy, Asthma and Immunology, 2010 Annual Meeting, New Orleans, USA, 2010.

IV National Spanish Congress of Clinical Laboratory of Medicine, Zaragoza, Spain, 2010.

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Manuscript received February 8, 2013; accepted for publication April 19, 2013.

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