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A new *PTGDR* promoter polymorphism in a population of children with asthma

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Recently, functional genetic variants of the *PTGDR* gene have been associated with asthma. The objective of this work was to study polymorphisms of the promoter region of *PTGDR* and their haplotype and diplotype combinations in a Spanish population of children with asthma. In this study, 200 Caucasian individuals were included. Asthma was specialist–physician diagnosed according to the ATS criteria. The polymorphisms were analyzed by direct sequencing. In the study, the new polymorphism (-613C > T) in the promoter region of *PTGDR* was analyzed. The CT genotype was more common in controls (17%) than in patients with asthma (1%) (p-value = 0.0003; OR, 0.057; 95% CI, 0.007–0.441). The CCCT CCCC diplotype (promoter positions -613, -549, -441, and -197) was more frequent in the group of patients with asthma [Fisher's p-value = 0.012; OR, 10.24; 95% CI (1.25–83.68)]; this diplotype is unambiguous. To our knowledge, this is the first study of -613C > T *PTGDR* polymorphism in patients. This analysis provides more complete information on influence of diplotype combinations of *PTGDR* polymorphisms in asthma.

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Scientists have succeeded in defining the molecular basis of some monogenetic diseases (1), but the genetic approach of complex diseases as asthma is much more difficult to establish. In this sense, new studies on the genetic contribution to asthma susceptibility are offering a new and amazing view of the asthma pathogenesis and opening ways for its treatment.

Asthma is the most common chronic condition in children and the main cause of hospitalization. It may affect as many as one in four urban children (2). This illness is characterized by inflammation of the bronchi resulting in the temporary narrowing of the airways. This inflammation is due to the secretion of different mediators that result in the accumulation of Th2 cells, eosinophils, and basophils. Among these mediators, prostaglandin D2 is the most abundantly produced cyclooxygenase metabolite of arachidonic acid in response to environmental allergens, and it has been proposed as a mast cell activation marker in asthma (3). Prostaglandin D2 elicits its biological responses by interaction with three specific seven-transmembrane receptors: D prostanoid receptor 1 (PTGDR1), D prostanoid receptor 2 (PTGDR2), and prostanoid TP receptor. PTGDR2 is a G-coupled receptor protein of 359 AA with a molecular mass of 40.27 kDa encoded by a gene located in chromosome 14q22 (4). Prostaglandin D2 receptor has recently been associated with asthma and atopy because of its gene location (5, 6) and the results obtained in functional studies performed in murine models. Thus, it has been observed that deletion of *PTGDR2* gene inhibits the airway inflammatory response to allergen (7). In addition, a highly selective PTGDR2 antagonist is effective in a murine model of allergic airway inflammation (8). Association of different polymorphisms in promoter region of *PTGDR* and asthma phenotype has been previously described (9–11) in adults. However, this

association has not been confirmed in certain populations (12, 13) and some inconsistencies have been observed regarding to the specific polymorphisms associated to the different asthma phenotypes (9, 10, 14).

In this work, we studied a population of children to find a putative association between the asthma phenotype and the previously described -197T > C, -441C > T, and -549T > C polymorphisms as well as -613C > T polymorphism located in the promoter region of the *PTGDR* gene.

Methods

Study populations

A total of 200 non-consanguineous Caucasian individuals were recruited in the Allergy Department of University Hospital of Salamanca (Spain): 86 children with asthma between 6 and 16 yr of age, and 114 adult controls. The study was performed after the approval and following the recommendations of the Hospital's Ethical Committee.

Data were collected from children diagnosed with asthma in the pediatric allergology outpatient clinic, presenting at least two symptoms of asthma (cough, wheezing, and dyspnea) in the last 12 months, and three asthma acute exacerbations documented in their case history, in the absence of other pulmonary disease. Children were at least 6-year old to perform a spirometry. All patients should have a positive bronchodilator test.

To confirm lack of development of asthma and allergy during childhood, a population of adult controls was selected. Healthy controls meeting the following criteria were included: (i) age over 18 yr, (ii) no symptoms or history of bronchial asthma or other respiratory diseases, (iii) absence of chronic skin diseases, (iv) no symptoms or history of allergy, (v) absence of first-degree relatives with asthma or atopia, and (vi) negative skin prick tests (< 1 mm wheal greater than saline) to the same battery of common aeroallergens.

Skin prick testing was performed to all patients and controls following the European Academy of Allergy and Clinical Immunology (EAACI) recommendations (15) with a battery of common aeroallergens previously described (10). Histamine 10 mg/ml was used as positive control and saline was used as negative control. Before skin testing, antihistamines were discontinued according to published guidelines. The allergic status was based on the positive skin test to at least one allergen. Skin tests were considered positive if at least one wheal reaction of more than 3 mm of diameter after subtraction of the negative control was observed. Total serum IgE levels were measured by a fluorenzymeimmunoassay (Pharmacia Cap System, Pharmacia, Uppsala, Sweden), following manufacturer's instructions.

Genotype and haplotype determination

Genomic DNA of patients was extracted from total blood with the DNA Extraction Kit (Genedan, S.L, Barcelona, Spain). The region of interest on the *PTGDR* promoter was amplified by polymerase chain reaction (PCR) in a MWG-BIOTECH thermal cycler (Ebersberg, Germany). PCRs were performed in a final volume of 25 μl including 1.25 pmol of each of the forward (5′-CTCAGTTTCCTCGCCTATGC-3′) and reverse (5′-ACCCCTGGAAGCCTACAACTGCAT-3′) primers, 20 ng of genomic DNA, and 12.5 μl of the commercial PCR Master Mix (Promega, Madison, WI, USA) containing Taq polymerase, dNTPs, and $MgCl_2$. To check contaminations, controls without genomic DNA were included in each PCR round of reactions. PCR products were cleaned up with the GENECLEAN Turbo Kit (Q-BIOgene, Cleveland, Ohio, USA), visualized on a 2% agarose gel stained with ethidium bromide, and sequenced in a 3100 Genetic Analyzer (Applied Biosystems, CA, USA) with the above described oligonucleotides. The sequence data and chromatograms were analyzed with the software CHROMASPRO (2003–07; Technelysium Pty Ltd, Tewantin, Australia). The program ALINGX of the VECTOR NTI 10 software (Invitrogen, Carlsbad, California, USA) was used for the alignment of all the obtained sequences. The GenBank accession number for the reference genomic sequence used for *PTGDR* alignments was AL355833.4; GI: 13990340.

The European Molecular Genetics Quality Network (EMQN) best practice guidelines (16, 17) were followed in all laboratory procedures as sample reception and storage. Special care was taken to minimize the risk of contamination during DNA extraction, the PCR reaction, and the cleanup of the PCR products; thus, the number of tube-to-tube transfers was minimized, ready-made solutions from commercial manufacturers were routinely employed, separate pre- and post-PCR areas were established, and cabinets and filter tips for pipettes were utilized. Sequencing method was chosen to genotype samples because of its liability. Control and patients were not genotyped in separated batches. The analysis was performed

blindly with respect to case–control status. Samples were double genotyped with a 100% of concordance, and all these results were replicated according to previously reported recommendations (18).

Statistics analysis

The SPSS software, version 12.0 (SPSS Inc., Chicago, IL, USA) and the SHESIS software platform (19) were employed for all statistical analyses. False positive report probability (FPRP) (20) and statistic power (21) were also calculated. The case–control study was performed by comparing the allele and genotype frequencies of patients to the control non-asthmatic group. For each SNP, the chi-square test was employed in the determination of the Hardy–Weinberg equilibrium. The chi-square test, the Fisher exact test, and the Monte Carlo simulation (after 10^4 simulations) were performed for the analysis of the dichotomous variables. The continuous variables were analyzed by the ANOVA test across the levels of each genotype. The effects of multiple covariates were modeled by logistic regression. Sex was included as potential covariate in multivariate analysis. A p-value of less than 0.05 was considered statistically significant. Bonferroni correction for multiple comparisons was applied when required. The web-based SNP analyzer (22) was employed for the haplotype estimation with the expectation–maximization-based algorithm. For the haplotype analysis, all haplotypes with a frequency of more than 1% among either patients or controls were considered; this analysis was performed using Monte Carlo simulation test, normal chi-square test, and odds ratio test. In addition, the chi-square test was applied for analysis of each haplotype vs. all other combined, for differences in haplotype frequencies between the case and control groups.

Results

Skin tests were positive in 94.5% of children and none of controls. Children have positive skin tests to pollens (69.1%), mites (40%), fungi (20%), and epithelia (28.7%). Mean percentage of basal spirometric values was 86.26% for FVC, 87.86% for FEV1, and 81.02% for FEF 25%–75%. Mean IgE value was 717.68 ± 1064.17 kU/l with a median value of 312 kU/l. There were significant differences between patients and controls (data not shown).

Table 1. Genotype and allele frequencies of -613C > T, -549T > C, -441C > T and -197T > C polymorphisms, according to asthma phenotype

		Genotype			Allele	
	n	TT	TC	CC	T	C
613C > T						
Controls	114	0.00	0.17	0.83	0.08	0.92
Asthma	86	0.00	0.01†	0.99†	0.01	0.99
549T > C						
Controls	114	0.20	0.52	0.28	0.46	0.54
Asthma	86	0.18	0.55	0.27	0.46	0.54
441C > T						
Controls	114	0.05	0.37	0.58	0.24	0.76
Asthma	86	0.09	0.36	0.55	0.27	0.73
197T > C						
Controls	114	0.81	0.12	0.07	0.87	0.13
Asthma	86	0.75	0.24	0.01‡	0.87	0.13

†For genotype frequencies, Fisher's p-value = 0.0003, Monte Carlo p-value (after 10^4 simulations) = 0.0003; for allelic frequencies, Fisher's p-value = 0.0004, Monte Carlo p-value (after 10^4 simulations) = 0.0004; ‡For genotype frequencies, Fisher's p-value = 0.017, Monte Carlo p-value (after 10^4 simulations) = 0.016.

Allele and genotype frequencies are presented in Table 1. We found that the genotype distribution of the -197T > C polymorphism seems to be associated with asthma [Fisher's p-value = 0.017, Monte Carlo p-value (after 10^4 simulations) = 0.016; Table 1].

Regarding the SNP identified in position -613C > T of the promoter region (Fig. 1), the CT genotype was less common in patients with asthma (1%) than in controls (17%) (p-value = 0.0003; OR, 0.057; 95% CI, 0.007–0.441) (Table 1). Multivariate analysis of the genotypes adjusted by sex confirmed this association with a

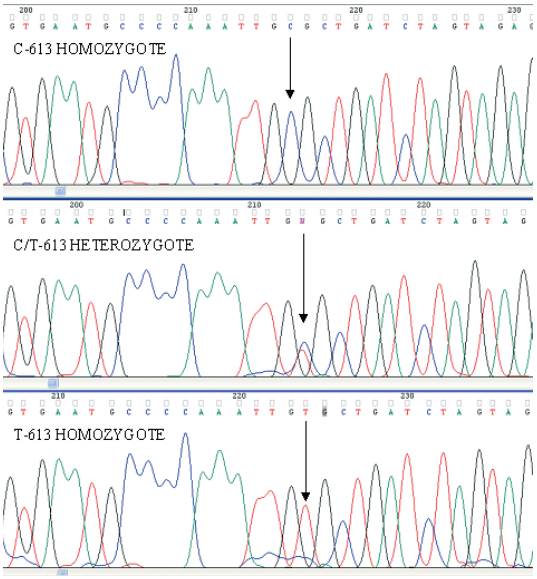

Fig. 1. Electropherogram -613C > T polymorphism.

Table 2. Haplotype frequencies of *PTGDR*

Haplotypes†	Controls (n = 114)	Asthma (n = 86)
CCCC	0.12	0.12
CCCT	0.30	0.39
CCTT	0.03	0.02
CTCT	0.25	0.21
CTTT	0.21	0.25
TCCT‡	0.07	0.01

Only haplotypes with a frequency of >1% among either controls or patients were included. The order of SNPs in the haplotype was -613C > T, -549T > C, -441C > T, and -197T > C. †Fisher's p-value for the general haplotype distribution = 0.028; ‡Fisher's p-value = 0.002 comparing the TCCT haplotype against all other haplotypes.

decreased risk of asthma (OR, 0.049; 95% CI, 0.006–0.391; p-value = 0.004). The FPRP of this association is less than 0.08 for a pre-test probability of 10%.

Haplotype frequencies in patients and controls are shown in Table 2. Differences in haplotype distribution were observed. A separate analysis of the TCCT haplotype against all other haplotypes showed a significant difference p-value = 0.002 (OR, 0.076; 95% CI, 0.010–0.578). This haplotype was less common in patients with asthma (0.01) than in controls (0.07). No differences were observed for the rest of haplotypes individually considered.

In the diplotype analysis, there was a difference in diplotype frequencies comparing patients with asthma and controls (p-value = 0.016; Table 3); this accounted specifically for the CCCT CCCC diplotype (-613CC, -549CC, -441CC, and -197TC). A separate analysis of this diplotype showed a specific association with asthma (p-value = 0.012; OR, 10.24; 95% CI, 1.25–83.68). Multivariate analysis of the diplotype adjusted by sex confirmed this association with an increased risk of asthma (OR, 9.907; 95% CI, 1.169–83.967; p-value = 0.035).

Table 3. Diplotype frequencies of *PTGDR*

Diplotypes†	Controls (n = 114)	Asthma (n = 86)
CCCC CCCC	0.06	0.01
CCCT CCCC‡	0.01	0.09
CCCT CCCT	0.08	0.13
CCCT CTCT	0.15	0.19
CCCT CTTT	0.14	0.21
CCCT TCCT	0.07	0.01
CTCT CTTT	0.10	0.07
CTTT CCCC	0.03	0.07
CTTT CTTT	0.03	0.06

Diplotypes with a frequency of >5% among either controls or patients were included. The order of SNPs in the haplotype was -613C > T, -549T > C, -441C > T, and -197T > C. †Fisher's p-value for the general diplotype distribution = 0.016; ‡Fisher's p-value = 0.012 comparing CCCT CCCC diplotype against all other diplotypes.

Discussion

The prostanoid DP receptor has been linked to asthma because of its gene location and to functional studies. It has been demonstrated that mice without a functional *PTGDR* receptor have less signs of inflammation in the lungs, do not develop airway hyper-responsiveness, and show marginal eosinophil infiltration (7). In this study, we analyzed for the first time the new polymorphism (-613C > T) in the promoter region of the *PTGDR* gene in children. We found that the T allele was significantly less frequent in patients with asthma. In addition, association of the C allele of -197T > C polymorphism with asthma was detected. This last association was previously described in adults (10) although different results have been reported in different ethnic populations (12).

Combinations of these polymorphisms were analyzed in a diplotype study. The CCCT CCCC diplotype (-613CC, -549CC, -441CC, and -197TC) was more frequent in patients with asthma. Almost 90% of children who carried the CCCT CCCC diplotype were asthmatic. The CCT and CCC haplotypes (-549CC, -441CC, and -197TC) are the two highest transcriptional efficiency haplotypes described so far (9). The inclusion of this polymorphism -613C > T provides interesting additional information.

Polymorphisms in the promoter region of *PTGDR* gene have been associated with asthmatic phenotype through transcription regulation. The differences in transcriptional efficiency are explained by the associated differences in transcription factor binding (9). In this sense, -613 and -197 SNPs are located in the binding site of different transcription factors; some of them involved in airway smooth muscle cell proliferation (23). Different modifications on sequence of *PTGDR* promoter in specific binding sites could, consequently, affect the transcription regulation of the gene, determining differences in asthma susceptibility. In this sense, *PTGDR* is pointed as a putative therapeutic target and new *PTGDR* antagonists are now being considered (8, 24).

Genetic association studies must be carefully considered. Many factors may influence on positive results. The classification of phenotypes is critical. In this study, criteria for selection of controls were very restrictive and the asthma phenotype was carefully classified following proper criteria.

Another crucial aspect of association studies is the application of quality control measures in laboratory procedures. In this work, EMQN good practice guidelines were followed. SNPs were retyped and closely scrutinized to ensure the correct genotyped, and the analysis was performed blindly with respect to control–patient status.

In addition, stringent significant levels, and statistical quality control are required to assure reproducibility (25). To limit type I error, the number of empirical analyses was limited and very stringent significant levels were considered to assure reproducibility. In addition, the effect of multiple comparisons was controlled and a specific analysis of false positive report probability was included. The effect of possible covariates, such sex was also analyzed by logistic regression. The sample size was also particularly considered as the statistical power depends directly on this aspect. Considering our population as reference, the statistical power of this study for the new polymorphism is more than 90% for an alpha error of 0.05, what implies that although limited, this sample size seems to be enough to show the trend of association; however, diplotype analysis would require a higher population to confirm results. *In vitro* studies on transcription levels of the haplotypes (9) and previous studies in adult population (10) are consistent with the results.

Bias in association studies can be observed because of the individual approach to the analysis of polymorphism. To avoid this situation, a more complete analysis of genetic combinations, including haplotype and diplotypes, was performed. This analysis provided additional information that can help to explain inconsistence in previous studies. In spite of these considerations, inconsistencies in association studies can be observed (12). Penetrance of the different alleles may be due to other factors. Considering the genetic heterogeneity, the frequency of SNPs can differ between ethnic groups.

The true model of genetic susceptibility for diseases, such as asthma, is complex (26, 27). In this scenery, contribution of association studies should be taken into account. We analyzed a new polymorphism with interesting genetic associations. To the best of our knowledge, this is the first study of *PTGDR* gene in European children.

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