Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

EAACI

Allergy

ORIGINAL ARTICLE EPIDEMIOLOGY AND GENETICS

PTGDR gene in asthma: a functional, genetic, and epigenetic study

M. Isidoro-García[1,2*], C. Sanz[3*], V. García-Solaesa[1], M. Pascual[4], D. B. Pescador[4], F. Lorente[4] & I. Dávila[4]

[1]Department of Clinical Biochemistry, University Hospital of Salamanca; [2]Department of Medicine, University of Salamanca; [3]Department of Microbiology and Genetics, University of Salamanca; [4]Department of Allergy, University Hospital of Salamanca, Salamanca, Spain

To cite this article: Isidoro-García M, Sanz C, García-Solaesa V, Pascual M, Pescador DB, Lorente F, Dávila I. *PTGDR* gene in asthma: a functional, genetic, and epigenetic study. *Allergy* 2011; **66**: 1553–1562.

Keywords
electrophoretic mobility shift assays; epigenetic; expression; polymorphism; promoter.

Correspondence
María Isidoro García, Department of Clinical Biochemistry, University Hospital of Salamanca, Paseo de San Vicente 58, 37007 Salamanca, Spain.
Tel.: 34 923 291373
Fax: 34 923 294593
E-mail: misidoro@usal.es

*These authors contributed equally to this study.

Accepted for publication 16 July 2011

DOI:10.1111/j.1398-9995.2011.02685.x

Edited by: Stephan Weidinger

Abstract

Background: Asthma affects more than 300 million individuals in the world. Several studies have demonstrated the importance of the genetic component. The aim of this study is to develop a holistic approach, including genetic, epigenetic, and expression analysis to study the Prostaglandin D2 receptor gene (*PTGDR*) in asthmatic patients.

Methods: In this study, 637 Caucasian individuals were included. Genetic variants were characterized by sequencing, and haplotype and diplotype combinations were established. Electrophoretic mobility shift assays (EMSAs) were performed with different promoter variants. An epigenetic analysis of *PTGDR* was for the first time developed by MassArray assays, and gene expression was determined by real-time polymerase chain reaction.

Results: The −197T > C (Fisher's $P = 0.028$) and −613C > T (Fisher's $P < 0.001$) polymorphisms were found to be significantly associated with allergic asthma and allergy to pollen and mites, respectively. In addition, several haplotype and diplotype combinations were associated with different allergy and asthma phenotypes. The presence of the −613C > T SNP determined variations in the EMSAs. Moreover, consistent differences in the methylation and expression patterns were observed between asthmatic patients and controls determining a 2.34-fold increase of PTGDR gene expression in asthmatic patients.

Conclusions: Genetic combinations described have functional implications in the *PTGDR* promoter activity by changing the transcription factors affinity that will help characterize different risk groups. The differences observed in the transcription factors affinity and in the methylation pattern bring insight into different transcription regulation in these patients. To the best of our knowledge, this is the first work in which the implication of genetic and epigenetic factors of *PTGDR* has been characterized pointing to putative therapeutic targets.

Asthma is an inflammatory disorder of the airways, which is thought to be caused by the interaction of multiple genes and environmental factors (1, 2). T cells and immunoglobulin E (IgE)-mediated responses are key factors in the development of allergic asthma. Prostaglandin D2 (PGD2) is the most abundantly produced cyclooxygenase metabolite of arachidonic acid in response to environmental allergens and has been proposed as a mast cell activation marker in asthma (3). Allergen exposure increases the *de novo* production of PGD2 in allergic patients causing bronchoconstriction, vasodilatation, increased capillary permeability, or mucous production (4). PGD2 exerts its function mainly through two specific receptors, the D prostanoid receptor (PTGDR or DP) (5) and the D prostanoid receptor 2 (6). Prostaglandin D2 or DP-specific agonists inhibit apoptosis, prolong the eosinophil survival, and block the interleukin 12, biasing the development of naive T lymphocytes to Th2 cells (7, 8). The proinflammatory role of DP has been further supported in guinea-pig models (9).

Prostaglandin D2 receptor gene is located on chromosome 14q22.1 (5), a region repeatedly linked to asthma (10–12). Genetic association studies have found significant association

between DP SNPs and asthma (13–17). Since Oguma et al. (14) firstly reported the associations, their observations have been reproduced in different Caucasian populations (13–15) although not in others (18, 19), suggesting differences among ethnic groups (18).

The alarming increase in the prevalence of allergic diseases in developed countries might have an important epigenetic component. However, the information about epigenetic studies in allergic diseases is scarce (2, 20). It is possible that the combination of genetic and epigenetic factors modulates the expression of *PTGDR* gene; therefore, a holistic approach is needed for a deeper comprehension of the pathological mechanisms of the disease. In this sense, we decided to study in parallel the putative association between asthma and different polymorphisms in the promoter region of the *PTGDR* gene, and the molecular mechanisms by which the presence of these polymorphisms results in functional changes including promoter affinity studies, expression assays and for the first time the epigenetic state analysis of the *PTGDR* promoter region.

Methods

Study population

A total of 637 Caucasian individuals (Table 1) were included. The approval of the Ethical Committee of the University Hospital of Salamanca and informed written consent from study subjects were obtained. The physician-diagnosed asthma criteria included the following: at least two symptoms consistent with asthma; either bronchial hyperreactivity, defined by a positive methacholine or a positive bronchodilator tests; and the absence of other pulmonary disorders. Lung function was measured following the American Thoracic Society criteria (21). Controls strictly fulfilled the following criteria: (i) no symptoms or history of asthma or other pulmonary diseases; (ii) no symptoms or history of allergy; (iii) negative skin prick tests to a battery of common aeroallergens (16, 22); and (iv) the absence of first-degree relatives with a history of asthma or atopy.

Genotype and haplotype analysis

DNA purification, polymerase chain reaction (PCR) amplification, and sequencing were performed as previously described (15); primers are described in Table S1 in supporting information. Chromas 2.3 (Technelysium, Tewantin, Australia) and AlingX software (Invitrogen, Carlsbad, CA, USA) were employed for the analysis. Specific quality measures were taken in all procedures following the EMQN guidelines (23).

Gel retardation-shift assay and *in silico* characterization of transcription factors

DNA–protein interactions were analyzed using DIG Gel Shift kit (Roche, Indianapolis, IN, USA). *In silico* studies considering −613C > T (rs_34236606), −549T > C (rs_8004654), −441C > T (rs_803010), −197T > C (rs_11157907), and −95G > T (described in this work) variations were performed to analyze transcription-factor-binding sites using ElDorado (Genomatix Software GmbH, http://www.genomatix.de/en/index.html) and the Transcription Element Search System 'TESS' (http://www.cbil.upenn.edu/tess/techreports/1997/CBIL-TR-1997-1001-v0.0.pdf).

Methylation analysis

A sample of 36 individuals (18 patients with allergic asthma and 18 controls) was selected following the criteria described in the Study Population section. The methylation analysis was firstly performed in the 36 individuals, and a confirmatory assay was developed in a group of six individuals. To avoid any epigenetic variation because of current presence or absence of allergen stimulation (seasonal allergy) in this population, patients with house dust mite allergy (an allergen permanently present in the environment) were selected. To avoid Th1/Th2 variation because of the allergic status of the selected sample, a B CD19+ lymphocytes cell population was selected for methylation and expression analyses. B lymphocytes were extracted from peripheral blood mononuclear cells by immune-magnetic positive selection (Invitrogen). Bisulfite conversion of genomic

Table 1 Phenotypic characteristics of the study population

Characteristic	Population	Controls	Asthma	Atopy	Atopic Asthma
Number of subjects	637	251	351	297	262
Sex (*n*) (%)					
Female	59	61	59	53	56
Male	41	39	41	47	44
Age					
Geometric Mean ± SD	40.49 ± 19.21	48.41 ± 19.08	35.22 ± 17.55	30.87 ± 15.61	30.19 ± 15.35
Log IgE					
Geometric Mean ± SD	1.95 ± 0.66	1.57 ± 0.58	2.23 ± 0.59	2.31 ± 0.56	2.35 ± 0.54
Allergen (%)					
Pollen	28.9	0	52.4	61.9	70.2
Mite	21.7	0	39.3	46.5	52.7
Pollen + Mite	9.7	0	17.7	20.9	23.7
Epithelia	10.7	0	19.4	22.9	25.9
Fungi	4.4	0	7.9	9.4	10.7

DNA was performed using the EZ DNA Methylation kit (Zymo, Irvine, CA, USA). Primers were designed using MethPrimer (http://www.urogene.org/methprimer/) and checked for specificity using BiSearch (http://bisearch.enzim.hu/). The primers were adapted for MassArray analysis (Table S1 in the Supporting Information). Polymerase chain reactions were carried out using FastStart High Fidelity kit (Roche). The percentage of the single CpG methylation was obtained using MALDI-TOF mass spectrometry (MassArray) provided by Sequenom, in duplicate.

Expression analysis

The same six individuals studied in the methylation assays were submitted to an expression analysis, in which *PTGDR* mRNA levels were determined by qPCR. Total RNA was extracted from CD19+ lymphocytes using TRIzol (Invitrogen). cDNA was generated using the SuperScript III kit (Invitrogen), and qPCR was performed in triplicate using a LightCycler® 480 Instrument and SYBR Green I Master Mix (Roche). Results were analyzed by the Livak method, normalized to *GAPDH* expression, and expressed as fold change difference between groups.

Statistical analysis

The chi-square test, Fisher's exact test, and Monte Carlo (10^4 simulations) were performed for the dichotomous variables; ANOVA test was employed for continuous variables across each genotype. Hardy–Weinberg equilibrium was evaluated. Logistic regression was employed to model the effects of multiple covariates, including potential covariates. Haplotype interactions were analyzed by Shesis (24) and the nonparametric multifactor dimensionality reduction (MDR) (25). The EM-based algorithm from the web-based SNPanalyzer (26) was employed for the diplotype estimation. Correction for multiple comparisons, false-positive report probability (FPRP), and statistic power were also calculated.

Results

Identification of sequence variants

A new polymorphism −95G > T was detected within a restriction site recognized by *AvaI* and its isoschizomer *BsoBI* (Fig. S1 in the Supporting Information). *AvaI* is methylation-sensitive whereas *BsoBI* is not, which simultaneously allows an epigenetic analysis of the region. The −95G > T SNP is in strong linkage disequilibrium with −549T > C, −441C > T, and −197T > C polymorphisms (D′ = 0.99, 0.99, and 0.94, respectively), but not with −613C > T (D′ = 0.01). Linkage disequilibrium analysis is shown in Fig. S3 in the Supporting Information.

Genetic association analysis

The −197T > C genotypic distribution was significantly associated with asthma and particularly with allergic asthma (Table 2). Multivariate analysis of the genotypes adjusted for age and sex confirmed this association with an increased risk of having asthma (OR, 5.94 95% CI 1.41–24.47; *P*-value = 0.014). Associations with log IgE levels (*P*-value < 0.001; Table 1) were also observed.

The allelic (additive model) and genotypic distributions of the −613C > T were significantly associated with allergy and mainly with allergy to both pollen and mites (Fisher's *P*-value < 0.001, Monte Carlo 10^4 = 0.001; Table 2).

In the haplotype analysis, a significant association between the general haplotype distribution and the group of allergic asthma to mite and pollen allergy was found (*P*-value = 0.034). An independent analysis of the TCCTG (−613T, −549C, −441C, −197T, and −95G) haplotype showed a significant association *P*-value = 0.005 (OR 2.39; 95% CI 1.28–4.46; Table 3). Considering only the four polymorphisms (−613C > T, −549T > C, −441C > T, and −197T > C) previously described, the association with the pollen and mite allergy was also observed for the general haplotype distribution (Fisher's *P*-value = 0.004), for the CTCT combination with a *P*-value = 0.041 (OR 0.58; CI 0.35–0.98) and for the TCCT combination with *P*-value < 0.001(OR 2.56; 95% CI 1.44–4.56); this result was confirmed with a statistical power > 80% for an α = 0.05 and with a FPRP of 5% for *a priori* probability of 0.1.

In the diplotype analysis, significant differences were observed in asthma (Fisher's *P*-value = 0.031) and allergic asthma (Fisher's *P*-value = 0.028). The TCCTG CTTTG diplotype combination was significantly associated (Fisher's *P*-value = 0.019 OR, 11.06; 95% CI 1.32–92.91) with pollen and mite allergy (Table 3). Both significant haplotype and diplotype associations were confirmed by the MDR analysis (Table S3 and Fig. S2 in the Supporting Information).

Effects of variants on DNA-binding proteins

Different transcription-factor-binding patterns were demonstrated in electrophoretic mobility shift assays (EMSAs) for genetic variants (Fig. 1). Differences in migration pattern of the −613C/G and −613T/A region: nuclear protein complexes were observed (Fig. 1). *In silico* study (Table S2 in the Supporting Information) showed transcription-factors-binding differences between wild and mutant alleles for this −613 position; however, certain differences were observed according to the platform employed; thus, the Eldorado analysis identified Zinc finger protein 336 (ZNF336), whereas CCAAT/enhancer-binding protein alpha (C/EBP alpha) was identified by TESS analysis.

DNA methylation and RNA expression

Using a quantitative mass-spectrometry-based method (27), we developed an exploratory analysis to interrogate the CpG methylation levels of allergic asthma and control subjects, focusing on three loci along the *PTDGR* promoter (Fig. 2). Individual information of six CpGs within the first amplicon was obtained. The second and the third amplicons were designed to interrogate 24 CpGs and 22 CpGs, respectively. *PTGDR* methylation patterns showed a decrease of methylation in allergic samples when compared to controls. Four

Table 2 Genotypic and allelic frequencies of *PTGDR* promoter SNPs

Phenotype	Genotype					Allele		
–613C>T	*n*	CC	CT	TT	*P*-value	C	T	*P*-value
Controls	251	0.84	0.16	0.00		0.92	0.08	
Asthma	351	0.81	0.18	0.01	0.17	0.90	0.10	0.17
Atopic Asthma	262	0.79	0.20	0.01	0.12	0.89	0.11	0.10
Family history of Asthma	156	0.84	0.16	0.00	0.91	0.92	0.08	0.91
Family history of Atopy	156	0.77	0.21	0.02	**0.027**	0.87	0.13	**0.024**
Pollens	184	0.76	0.23	0.01	**0.042**	0.87	0.13	**0.025**
Mites	138	0.77	0.21	0.02	**0.027**	0.87	0.13	**0.036**
Pollen and Mites	62	0.67	0.29	0.04	**<0.001**	0.82	0.18	**0.001**
–549T>C	*n*	TT	TC	CC	*P*-value	T	C	*P*-value
Controls	251	0.24	0.51	0.25		0.49	0.51	
Asthma	351	0.20	0.52	0.28	0.57	0.46	0.54	0.32
Atopic Asthma	262	0.28	0.52	0.20	0.51	0.54	0.46	0.27
Family history of Asthma	156	0.27	0.50	0.23	0.94	0.52	0.48	0.84
Family history of Atopy	156	0.29	0.50	0.21	0.67	0.54	0.46	0.38
Pollens	184	0.29	0.55	0.16	0.15	0.56	0.44	0.12
Mites	138	0.29	0.50	0.21	0.76	0.54	0.46	0.47
Pollen and Mites	62	0.13	0.55	0.32	0.09	0.41	0.59	0.035
–441C>T	*n*	CC	CT	TT	*P*-value	C	T	*P*-value
Controls	251	0.60	0.35	0.05		0.78	0.22	
Asthma	351	0.60	0.35	0.05	0.99	0.78	0.22	0.93
Atopic Asthma	262	0.60	0.35	0.05	0.99	0.77	0.23	1
Family history of Asthma	156	0.58	0.37	0.05	0.91	0.77	0.23	0.74
Family history of Atopy	156	0.63	0.32	0.05	0.86	0.79	0.21	0.59
Pollens	184	0.58	0.38	0.04	0.78	0.77	0.23	0.80
Mites	138	0.58	0.36	0.06	0.91	0.76	0.24	0.66
Pollen and Mites	62	0.61	0.37	0.02	0.81	0.80	0.20	0.57
–197T>C	*n*	TT	TC	CC	*P*-value	T	C	*P*-value
Controls	251	0.80	0.16	0.04		0.88	0.12	
Asthma	351	**0.77**	**0.22**	**0.01**	**0.016**	0.88	0.12	0.90
Atopic Asthma	262	**0.77**	**0.22**	**0.01**	**0.028**	0.88	0.12	0.97
Family history of Asthma	156	**0.72**	**0.27**	**0.01**	**0.009**	0.86	0.14	0.34
Family history of Atopy	156	**0.73**	**0.26**	**0.01**	**0.030**	0.86	0.14	0.39
Pollens	184	0.81	0.18	0.01	0.26	0.90	0.10	0.37
Mites	138	0.79	0.20	0.01	0.74	0.89	0.11	0.16
Pollen and Mites	62	0.82	0.18	0.00	0.31	0.91	0.09	0.24
–95G>T	*n*	GG	GT	TT	*P*-value	G	T	*P*-value
Controls	251	0.97	0.03	0.00		0.98	0.02	
Asthma	351	0.97	0.03	0.00	0.62	0.98	0.02	0.91
Atopic Asthma	262	0.96	0.03	0.01	0.62	0.98	0.02	0.75
Family history of Asthma	156	0.97	0.03	0.00	0.78	0.98	0.02	0.78
Family history of Atopy	156	0.95	0.05	0.00	0.60	0.98	0.02	0.61
Pollens	184	0.97	0.03	0.01	0.42	0.98	0.02	0.81
Mites	138	0.96	0.04	0.00	0.74	0.98	0.02	0.74
Pollen and Mites	62	0.96	0.04	0.00	0.91	0.98	0.02	0.91

Bold values indicate significant associations.

methylation measurements, PTG.1_CpG_6, PTG.2_CpG_11.12, PTG.2_CpG_17.18.19.20, and PTG.2_CpG_22 (Fig. 2), showed a statistically significant difference with a statistical power >80% for $\alpha = 0.05$ (Table 4). The results of the first methylation assay were confirmed with a second confirmatory analysis obtaining more than 98% of correlation between the two assays (Table S4 in the Supporting Information).

Table 3 Haplotype and Diplotype frequencies. Data represent the percentage of each haplotype or diplotype in each phenotypic group/Fisher's *P*-value. Haplotypes with a frequency >1% among either controls or patients are included

Haplotype	Control	Asthma	Pollen-Mite	Haplotype	Control	Asthma	Pollen-Mite
CCCTG	0.32	0.32/0.89	0.36/0.40	CCCT	0.31	0.32/0.80	0.37/0.24
CTCTG	0.27	0.23/0.14	0.18/0.06	CTCT	0.26	0.23/0.25	**0.17/0.041**†††
CTTTG	0.22	0.23/0.76	0.22/0.96	CTTT	0.22	0.22/0.90	0.19/0.44
CCCCG	0.09	0.10/0.80	0.05/0.17	CCCC	0.11	0.12/0.85	0.08/0.27
TCCTG	0.08	0.11/0.14	**0.17/0.005**†	TCCT	0.08	0.10/0.20	**0.18 /<0.001**††
CCCCT	0.02	0.01/0.62	0.01/0.60	CCCC	0.11	0.12/0.85	0.08/0.27
CTCCG	0.00	0.00/0.90	0.00/0.50	CTCC	0.01	0.01/0.90	0.00/0.50
TTTCT	0.00	0.00/1	0.01/0.18	TTTC	0.00	0.00/1	0.01/0.18
Diplotype	**Control**	**Asthma**	**Pollen-Mite**	**Diplotype**	**Control**	**Asthma**	**Pollen-Mite**
CTCTG CCCTG	0.21	0.17/0.32	0.16/0.56	CTCT CCCT	0.20	0.17/0.32	0.15/0.56
CTTTG CCCTG	0.12	0.16/0.32	0.18/0.36	CTTT CCCT	0.13	0.15/0.32	0.15/0.36
CTTTG CTCTG	0.11	0.09/0.56	0.06/0.43	CTTT CTCT	0.11	0.09/0.56	0.05/0.43
CCCTG CCCTG	0.10	0.08/0.45	0.12/0.80	CCCT CCCT	0.09	0.09/0.45	0.12/0.8
CTCTG CTCTG	0.07	0.05/0.58	0.04/0.75	CTCT CTCT	0.06	0.05/0.58	0.03/0.75
TCCTG CCCTG	0.07	0.09/0.72	0.10/0.38	TCCT CCCT	0.06	0.05/0.72	0.14/0.38
CTTTG CTTTG	0.05	0.05/1	0.02/0.47	CTTT CTTT	0.05	0.05/1	0.02/0.47
CCCCG CTTTG	0.05	0.05/1	0.04/1	CCCC CTTT	0.05	0.05/0.85	0.03/1
TCCTG CTTTG	0.04	0.05/0.84	**0.14/0.019***	TCCT CTTT	0.04	0.05/0.84	0.12/0.021
CCCCG CTCTG	0.04	0.03/0.64	0.04/1	CCCC CTCT	0.05	0.05/0.28	0.05/1
CTCTG TCCTG	0.04	0.05/0.41	0.02/1	CTCT TCCT	0.03	0.05/0.41	0.02/1
CCCCG CCCTG	0.03	**0.08/0.018****	0.02/1	CCCC CCCT	0.04	**0.09/0.008**‡	0.05/0.67
CCCCG CCCCG	0.03	**0.00/0.004*****	0.00/0.59	CCCC CCCC	0.04	**0.01/0.019**‡‡	0.00/0.36
CCCCG TCCTG	0.00	0.03/0.054	0.02/0.33	CCCC TCCT	0.02	0.03/0.37	0.03/0.56
TCCTG TCCTG	0.00	0.01/0.27	0.02/0.18	TCCT TCCT	0.00	0.01/0.27	0.02/0.18

†Fisher's *P*-value = 0.005; OR 2.39, 95%CI (1.28–4.46) comparing the haplotype against all others. Fisher's *P*-value for the general haplotype distribution in the Pollen and Mite Allergy group was 0.03. The order of the SNPs is −613C>T, −549T>C, −441C>T, 197T>C, and −95G>T.
††Fisher's *P*-value <0.001; OR 2.56, 95%CI (1.44–4.56) comparing the haplotype against all others. Fisher's *P*-value for the general haplotype distribution in the Pollen and Mite Allergy group was 0.004. The order of the SNPs is −613C>T, −549T>C, −441C>T and 197T>C.
†††Fisher's *P*-value = 0.041; OR 0.58, 95%CI (0.34–0.98) comparing the haplotype against all others.
*Fisher's *P*-value = 0.019; OR 3.44, CI (1.24–9.52) comparing the diplotype against all others.
**Fisher's *P*-value = 0.018; OR 2.70, CI (1.15–6.34) comparing the diplotype against all others. Fisher's *P*-value for the general diplotype distribution in the asthma group was 0.031.
***Fisher's *P*-value = 0.004; OR 0.40, CI (0.36–0.44) comparing the diplotype against all others.
‡Fisher's *P*-value = 0.008; OR 2.71, CI (1.27–5.77) comparing the diplotype against all others.
‡‡Fisher's *P*-value = 0.019; OR 0.21, CI (0.06–0.76) comparing the diplotype against all others.
Bold values indicate significant associations.

One differentially methylated region between allergic and control subjects, the PTG.1.6_CpG6, is located in the −613C > T SNP position. The methylation level in patients with −613CC genotype is clearly higher (84.2%) than in patients with CT genotype (42.5%); however, this difference is not observed in the control group where CC controls have a methylation level of 96.4%, and CT controls have 97.5% (Table S5 in Supporting Information). These results certainly suggest that the −613CT genotype may have some influence in the methylation level, but it does not seem to be the only factor affecting methylation because CT controls remain with a high methylation level.

The mRNA expression levels of PTGDR were checked in a small population of CD19+ lymphocytes of three patients and three controls. A 2.34-fold increase in the expression level of this gene was observed in allergic patients (Fig. 2, Table S4 in Supporting Information).

Discussion

In this study, a new polymorphism −95G > T and a simplified genotyping method of restriction analysis are provided. This new SNP allows us to study a more complete set of promoter polymorphisms. The −197T > C and −613C > T polymorphisms were significantly associated with asthma and allergy, respectively. The complete haplotype study provided an association of the **TCCTG** combination that was more common in allergic patients and in atopic asthma. This result adds valuable information to the previously reported studies in which the partial combinations **TCCT** (16) and **CCT** (14) were associated with asthma. Interestingly, the **CCT** haplotype has been described to be a high transcriptional efficiency haplotype with a high PTGDR expression (14). In addition, the **CTCTG** combination, which was more common in controls, contains the **TCT** combination described as a low

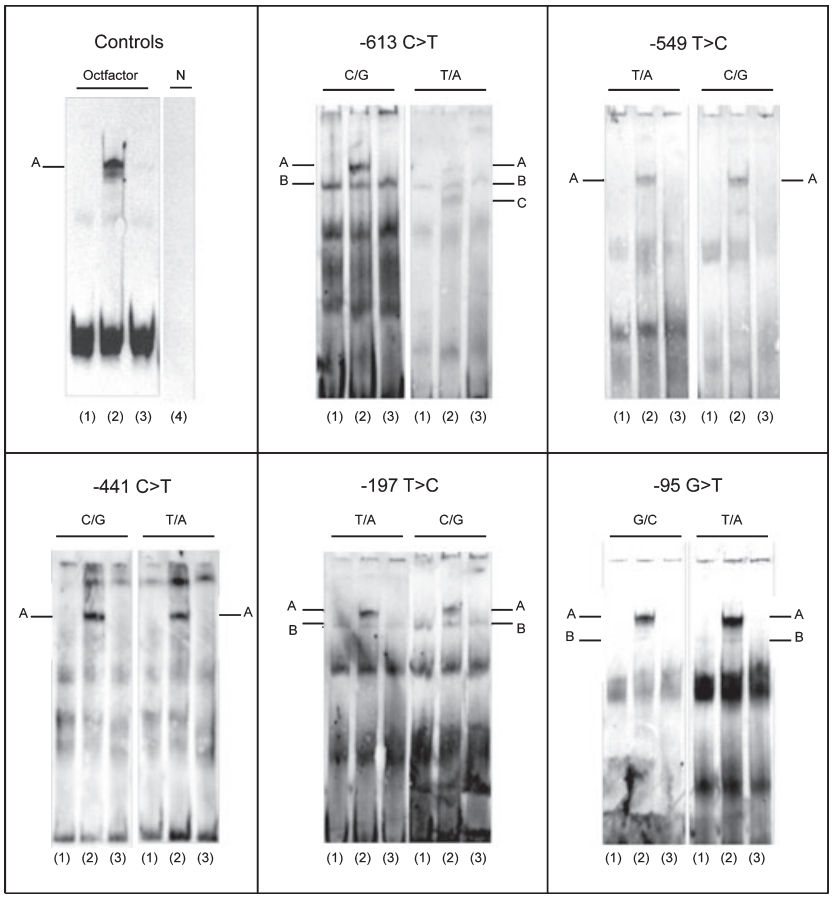

Figure 1 Electrophoresis mobility shift assays in −613C>T, −549T >C, −441C>T, −197T>C, and −95G>T positions of the *PTGDR* promoter. Study of the affinity effects of these nucleotide variations. (1) Labelled double strand (ds) oligonucleotide without nuclear proteins or unlabelled ds nucleotide. (2) Labelled ds oligonucleotide with nuclear proteins and without unlabelled ds oligonucleotide. (3) Labelled ds oligonucleotide with nuclear proteins and with unlabelled ds oligonucleotide (×25). (4) Negative control with only binding buffer.

transcriptional efficiency haplotype related to low PTGDR expression and a less predisposition to asthma (14).

Significant associations were also detected regarding the **C**CCCG C**CCT**G diplotype for asthma (Table 3) (15, 16). It is interesting to notice that it is a combination of the two CCC and CCT haplotypes that have been previously reported to be associated with a significantly higher PTGDR gene expression. To confirm our results, a new approach employing the MDR software was performed. The analysis redefined a single variable of high and low risk, combining information of several regions that may interact in disease etiology. The same haplotypes were confirmed as the best models.

These results are in agreement with previous studies (14, 15), although some differences have been reported in some Asian populations. Recently, a meta-analysis have shown that frequencies found in Caucasian, African-American, and Puerto-Ricans are similar (13) and could define a first ethnic group in which several *PTGDR*-Asthma associations have been described. The frequencies found in Asian and Mexicans are similar (28) and could define a second ethnic group, where *PTGDR* does not seem to be a candidate for asthma. Other aspects that may influence the controversy observed in these studies could be related to quality control measures, specifically, the selection criteria of the phenotypes. In this sense, strict criteria were defined to select the populations in this study. In addition, EMQN good practice guidelines were followed in all laboratory procedures, and stringent statistical quality controls were adopted (23), providing the FPRP, the statistical power, and the putative effect of potential covariates in logistic regression.

An important limitation to the majority of the susceptibility studies is that they are based only on descriptive analyses (18, 19). In this study, the putative functional implications of these SNPs were analyzed in the disease development. Using EMSAs, we detected that the −613C > T change gives rise to a modification in the transcription-binding affinity. The *in silico* analysis provided several transcription factors, specifically C/EBPα has been proposed as a pivotal factor in the generation of eosinophils, and it has been related to an equine model of asthma (29). In addition, the simultaneous activation of C/EBPα and glucocorticoid receptor (GR) might explain antiasthmatic effects of steroids and β-mimetic treatments, such as the reduced inflammatory cytokine levels. Modification in binding affinity can determine changes in the

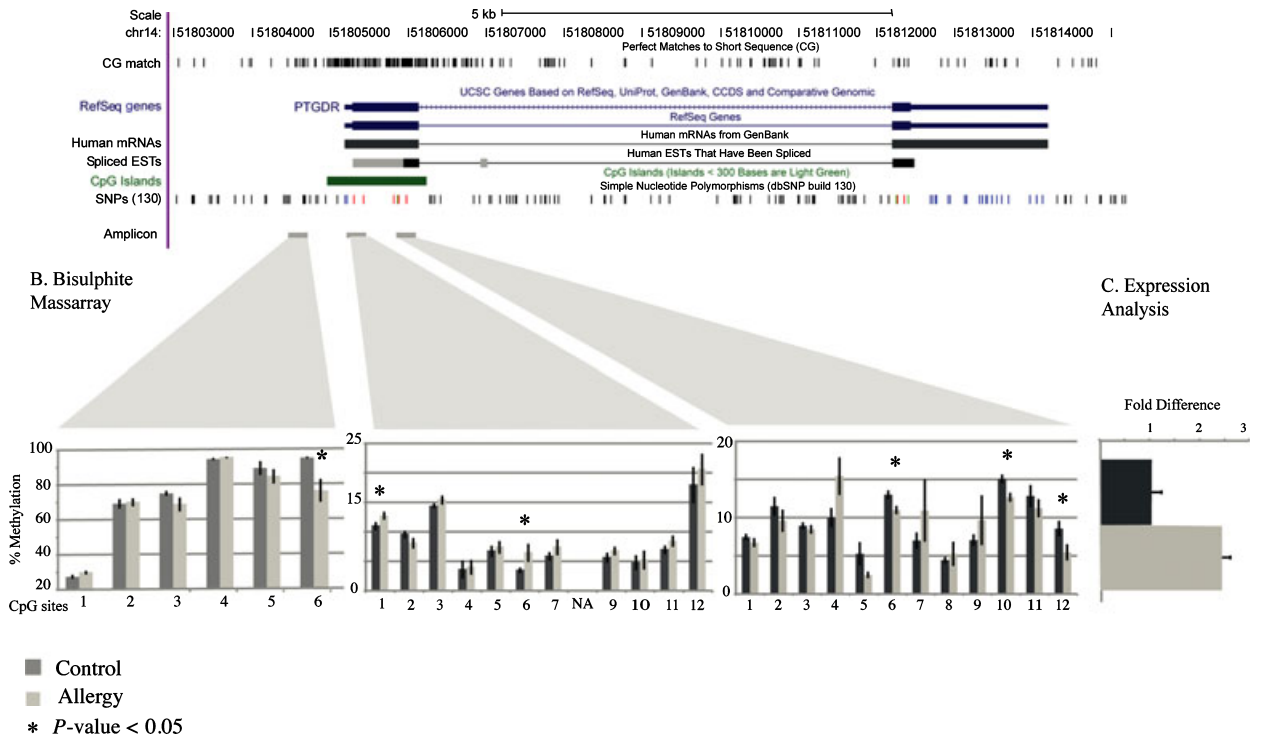

Figure 2 Methylation-expression analysis.

PTGDR gene expression, which can be associated with the disease development (14). What is more, individuals bearing low *PTGDR* expression haplotypes might be less susceptible to asthma because they less efficiently recruit critical subsets of lymphocytes to the airways and more efficiently resolve airway eosinophilia through apoptosis (30).

Other important mechanisms of gene expression regulation are epigenetic factors. In this study, we explore for the first time the DNA methylation levels along the *PTGDR* promoter, showing a distinctive DNA methylation pattern between controls and patients, in which allergic asthmatics show a hypomethylation of the promoter. This novel result is in agreement with the increase of the *PTGDR* expression detected in the mRNA analysis of B cells in these patients. One concern of this study is the small population for this exploratory gene expression analysis. In this sense, only results with a statistical power higher than 80% are presented (31). The important interaction between the genome and the environment in the susceptibility of inflammatory diseases has been demonstrated in some studies (32, 33); however, very little information about epigenetic studies in these diseases exists (2, 20). It has been shown that some environmental factors could determine epigenetic alterations of promoter sequences, with consequent modification of candidate genes. For this reason, integrative studies where genetic as well as epigenetic factors are considered could provide the real picture of these complex diseases.

The evidence that *PTGDR* is required for expression of the asthma in more than one animal model, its association with asthma susceptibility in humans, and the availability of safe and effective oral agents that inhibit the receptor provide a clear and compelling justification for human clinical trials (30). All together, our results would also point at this promoter region as a putative therapeutic target. New PTGDR antagonists are now being considered on allergy treatment (34–36), suggesting that selective prostanoid D2 receptor antagonists may provide beneficial effects for the clinical treatment of allergic diseases (37). The ability to identify individuals bearing a *PTGDR* haplotype that increases gene expression would allow trial designs that target interventions to individuals with greater expression and therefore greater expected benefits (30). However, in a recent study, a DP1 antagonist did not demonstrate efficacy in asthmatic patients or patients with allergic rhinitis (37). In the same study, variations in *PTGDR* did not appear related to treatment response. These clinical trials did not take into consideration the transcription factors (38) nor the epigenetic status that according to our results could influence the expression of *PTGDR* in asthmatic patients. In our results, one of the differentially methylated regions between patients and controls is located in the −613 position. This could open new insight in the analysis of therapeutic targets and could explain why certain individuals, with genetic variants that impaired *PTGDR* expression, develop asthma, what is consistent with the speculation that intense environmental exposures could overcome the protective effects of protective genotypes.

To our knowledge, this is the first study in which a new approach combining genetic, transcriptional, and epigenetic factors has been altogether considered in the *PTGDR* gene.

Table 4 Methylation analysis

	Controls		Allergics			
	Methylation %		Methylation %			
	Mean	SEM	Mean	SEM	*P*-value	Statistical power*
PTG.1.1_CpG_1 (−734 to −733)	27.42	1.16	29.81	1.04	0.15	
PTG.1.2_CpG_2 (−721 to −720)	69.33	2.71	70.19	2.32	0.82	
PTG.1.3_CpG_3 (−690 to −689)	75.33	1.41	68.94	3.97	0.22	
PTG.1.4_CpG_4 (−652 to −651)	95.00	0.64	95.88	0.40	0.25	
PTG.1.5_CpG_5 (−631 to −630)	89.83	3.96	85.06	4.14	0.44	
PTG.1.6_CpG_6 (−613 to −612)	**95.92**	**0.45**	**77.06**	**6.54**	**0.03**	**99.1**
PTG.2.1_CpG_1.2 (−179 to −176)	10.67	0.65	12.60	0.67	0.01	68.9
PTG.2.2_CpG_3 (−153 to −152)	9.00	0.61	8.05	0.90	0.47	
PTG.2.3_CpG_4.5.6 (−139 to −129)	14.40	0.35	15.40	0.78	0.16	
PTG.2.4_CpG_7 (−113 to −112)	3.36	1.60	3.97	1.25	0.71	
PTG.2.5_CpG_8.9 (−102 to −97)	6.20	0.89	7.37	1.08	0.31	
PTG.2.6_CpG_10.11.12.13 (−93 to −86)	3.46	0.50	6.43	1.53	0.03	73.5
PTG.2.7_CpG_14 (−78 to −77)	5.80	0.76	7.37	1.35	0.22	
PTG.2.8_CpG_15.16.17.18.19 (−60 to −36)	N/A		N/A			
PTG.2.9_CpG_20 (−8 to −7)	5.40	0.82	6.73	0.74	0.14	
PTG.2.10_CpG_21 (−4 to −3)	4.38	1.25	5.21	1.61	0.62	
PTG.2.11_CpG_22.23 (+8 to +12)	6.63	0.72	8.37	0.96	0.08	
PTG.2.12_CpG_24 (+19 to +20)	17.70	3.19	20.57	2.68	0.40	
PTG.3_CpG_1 (+567 to +568)	N/A		N/A			
PTG.3_CpG_2.3 (+578 to +579 & +584 to +589)	N/A		N/A			
PTG.3.1_CpG_4.5 (+623 to +624 & +636 to +637)	7.44	0.44	6.81	0.58	0.43	
PTG.3.2_CpG_6 (+642 to +643)	11.50	1.22	9.31	1.43	0.29	
PTG.3.3_CpG_7.8 (+657 to +658 & +660 to +661)	8.94	0.44	8.50	0.55	0.56	
PTG.3.4_CpG_9 (+667 to +668)	10.00	1.27	15.27	2.37	0.08	
PTG.3.5_CpG_10 (+680 to +681)	5.28	1.52	2.57	0.46	0.16	
PTG.3.6_CpG_11.12 (+688 to +689 &+691 to+692)	**13.00**	**0.58**	**11.00**	**0.52**	**0.02**	**94.1**
PTG.3.7_CpG_(+700 to +701)	7.00	1.06	10.25	3.99	0.46	
PTG.3.8_CpG_14.15 (+707 to +708 & +709 to +710)	4.44	0.42	5.13	1.53	0.69	
PTG.3.9_CpG_16 (+732 to +733)	7.06	0.77	9.31	3.15	0.52	
PTG.3.10_CpG_17.18.19.20 (+737 to +744)	**15.06**	**0.60**	**12.69**	**0.58**	**0.01**	**97.4**
PTG.3.11_CpG_21 (+747 to +748)	12.83	1.44	11.00	1.15	0.39	
PTG.3.12_CpG_22 (+758 to +759)	**8.56**	**1.02**	**5.25**	**1.03**	**0.04**	**88.9**

*Statistical power for α error 0.05.
Bold values indicate significant associations.

In this integrative work, we considered new promoter variants to define more specific haplotype and diplotype combinations related to allergy and asthma. We detected differences in the transcription-binding affinity, and we propose for the first time the consideration of epigenetic aspects of *PTGDR* gene, pointing to putative therapeutic targets. Further functional studies considering the complete *PTGDR* promoter are urgently needed to understand the molecular mechanism that relates *PTGDR* expression with the allergy and asthma development.

Acknowledgments

This work was supported by the Spanish Fondo de Investigación Sanitaria (FIS) grants PS09/02068 and PI10/01706, and by the Spanish Junta de Castilla y León Grants GRS205/A/08, GRS224/A/08, GR255 and HUS01A08. MP has the fellowship EDU/1708/2008. The authors thank Dr. John Greally for his assistance with the epigenetic and expression assays.

Author's contributions

All authors (MI-G, CS, VG-S, MP, DBP, FL, and ID) have contributed to the conception and design of this work, as well as to the acquisition, analysis and interpretation of data, to the drafting and critical revision of the manuscript and have approved the final version to be published.

Conflicts of interest

The authors of this work (MI-G, CS, VG-S, MP, DBP, FL, and ID) declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article found at: http://www.wileyonlinelibrary.com

Figure S1. −95G > T RFLP analysis.

Figure S2. MDR analysis.

Figure S3. Linkage disequilibrium (D′)

Table S1. Oligonucleotides used in this work.

Table S2. Results of *in silico* analysis with ElDorado and TESS platforms.

Table S3. MDR diplotype and haplotype analyses.

Table S4. Expression and methylation values.

Table S5. −613C > T SNP and methylation values in z1CpG6.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. Palmer LJ, Cookson WO. Genomic approaches to understanding asthma. *Genome Res* 2000;**10**:1280–1287.
2. Pascual M, Davila I, Isidoro-Garcia M, Lorente F. Epigenetic aspects of the allergic diseases. *Front Biosci (Schol Ed)* 2010;**2**:815–824.
3. O'Sullivan S. On the role of *PGD2* metabolites as markers of mast cell activation in asthma. *Acta Physiol Scand Suppl* 1999;**644**:1–74.
4. Alving K, Matran R, Lundberg JM. The possible role of prostaglandin D2 in the long-lasting airways vasodilatation induced by allergen in the sensitized pig. *Acta Physiol Scand* 1991;**143**:93–103.
5. Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. *J Biol Chem* 1995;**270**:18910–18916.
6. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor *CRTH2*. *J Exp Med* 2001;**193**:255–261.
7. Gervais FG, Cruz RP, Chateauneuf A, Gale S, Sawyer N, Nantel F et al. Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP. *J Allergy Clin Immunol* 2001;**108**:982–988.
8. Gosset P, Bureau F, Angeli V, Pichavant M, Faveeuw C, Tonnel AB et al. Prostaglandin D2 affects the maturation of human monocyte-derived dendritic cells: consequence on the polarization of naive Th cells. *J Immunol* 2003;**170**:4943–4952.
9. Arimura A, Yasui K, Kishino J, Asanuma F, Hasegawa H, Kakudo S et al. Prevention of allergic inflammation by a novel prostaglandin receptor antagonist, S-5751. *J Pharmacol Exp Ther* 2001;**298**:411–419.
10. Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR et al. Cookson WO: a genome-wide search for quantitative trait loci underlying asthma. *Nature* 1996;**383**:247–250.
11. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med* 2010;**363**:1211–1221.
12. Jamrozik EF, Warrington N, McClenaghan J, Hui J, Musk AW, James A et al. Functional haplotypes in the *PTGDR* gene fail to associate with asthma in two Australian populations. *Respirology* 2011;**16**:359–366.
13. Oguma T, Asano K, Ishizaka A. Role of prostaglandin D(2) and its receptors in the pathophysiology of asthma. *Allergol Int* 2008;**57**:307–312.
14. Oguma T, Palmer LJ, Birben E, Sonna LA, Asano K, Lilly CM. Role of prostanoid DP receptor variants in susceptibility to asthma. *N Engl J Med* 2004;**351**:1752–1763.
15. Sanz C, Isidoro-Garcia M, Davila I, Moreno E, Laffond E, Avila C et al. Promoter genetic variants of prostanoid DP receptor (*PTGDR*) gene in patients with asthma. *Allergy* 2006;**61**:543–548.
16. Sanz C, Isidoro-Garcia M, Davila I, de Pedro MP, Mendez SdeA, Padron J et al. A new *PTGDR* promoter polymorphism in a population of children with asthma. *Pediatr Allergy Immunol* 2009;**20**:151–156.
17. Zhu G, Vestbo J, Lenney W, Silverman M, Whyte M, Helms P et al. Association of *PTGDR* gene polymorphisms with asthma in two Caucasian populations. *Genes Immun* 2007;**8**:398–403.
18. Li J, Liu Q, Wang P, Li H, Wei C, Guo C et al. Lack of association between three promoter polymorphisms of *PTGDR* gene and asthma in a Chinese Han population. *Int J Immunogenet* 2007;**34**:353–357.
19. Tsai YJ, Choudhry S, Kho J, Beckman K, Tsai HJ, Navarro D et al. The *PTGDR* gene is not associated with asthma in 3 ethnically diverse populations. *J Allergy Clin Immunol* 2006;**118**:1242–1248.
20. Isidoro-Garcia M, Davila-Gonzalez I, Pascual de Pedro M, Sanz-Lozano C, Lorente-Toledano F. Interactions between genes and the environment. Epigenetics in allergy. Allergol Immunopathol (Madr) 2007;**35**:254–258.
21. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society. *Am J Respir Crit Care Med* 1995;**152**:S77–S121.
22. Skin tests used in type I allergy testing position paper. Sub-committee on skin tests of the European Academy of Allergology and Clinical Immunology. *Allergy* 1989;**44**(Suppl. 10):1–59.
23. Muller CR. Quality control in mutation analysis: the European Molecular Genetics Quality Network (EMQN). *Eur J Pediatr* 2001;**160**:464–467.
24. Shi YY, He L. Shesis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005;**15**:97–98.
25. Hahn LW, Ritchie MD, Moore JH. Multifactor Dimensionality Reduction software for detecting gene-gene and gene-environment interactions. *Bioinformatics* 2003;**19**:376–382.
26. Workbench J, Bonghee S, Yangseok K. SNP Analyzer: web based for the SNPs analysis. *Genome Inform* 2003;**14**:591–592.
27. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G et al. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc Natl Acad Sci U S A* 2005;**102**:15785–15790.
28. Leung TF, Li CY, Kong AP, Chan IH, Ng MC, Chan MH et al. PTGDR is not a major candidate gene for asthma and atopy in chinese children. *Pediatr Allergy Immunol* 2009;**20**:556–562.
29. Couetil LL, Art T, de Moffarts B, Becker M, Melotte D, Jaspar F et al. DNA binding activity of transcription factors in bronchial cells of horses with recurrent airway obstruction. *Vet Immunol Immunopathol* 2006;**113**:11–20.
30. Lilly CM, Palmer LJ. The role of prostaglandin D receptor gene in asthma patho-

genesis. *Am J Respir Cell Mol Biol* 2005;**33**:224–226.
31. Fleiss JL. Statistical methods for rates and proportions. New York: John Wiley & Sons 1973.
32. Kleeberger SR, Peden D. Gene-environment interactions in asthma and other respiratory diseases. *Annu Rev Med* 2005;**56**:383–400.
33. Upham JW, Holt PG. Environment and development of atopy. *Curr Opin Allergy Clin Immunol* 2005;**5**:167–172.
34. Uller L, Mathiesen JM, Alenmyr L, Korsgren M, Ulven T, Hogberg T et al. Antagonism of the prostaglandin D2 receptor CRTH2 attenuates asthma pathology in mouse eosinophilic airway inflammation. *Respir Res* 2007;**8**:16.
35. Shichijo M, Arimura A, Hirano Y, Yasui K, Suzuki N, Deguchi M et al. A prostaglandin D2 receptor antagonist modifies experimental asthma in sheep. *Clin Exp Allergy* 2009;**39**:1404–1414.
36. Stebbins KJ, Broadhead AR, Correa LD, Scott JM, Truong YP, Stearns BA et al. Therapeutic efficacy of AM156, a novel prostanoid DP2 receptor antagonist, in murine models of allergic rhinitis and house dust mite-induced pulmonary inflammation. *Eur J Pharmacol* 2010;**638**:142–149.
37. Philip G, van Adelsberg J, Loeys T, Liu N, Wong P, Lai E et al. Clinical studies of the DP1 antagonist laropiprant in asthma and allergic rhinitis. *J Allergy Clin Immunol* 2009;**124**:942–948.
38. Roth M, Black JL. Transcription factors in asthma: are transcription factors a new target for asthma therapy? *Curr Drug Targets* 2006;**7**:589–595.