ORIGINAL ARTICLE

Promoter Genotyping and mRNA Expression–Based Analysis of the *PTGDR* Gene in Allergy

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Abstract

Background: Prostaglandin D2 receptors are acquiring a relevant role as potential therapeutic targets in allergy. *PTGDR* has been described as a candidate gene in allergic disease, although functional studies on this gene are lacking.
Objective: The objective of this case-control study was to investigate the potential role of *PTGDR* in allergy.
Methods: The study population comprised 195 allergic patients and 112 healthy controls. The *PTGDR* promoter polymorphisms –1289G>A, –1122T>C, –881C>T, –834C>T, –613C>T, –549T>C, –441C>T, –197T>C, and -95G>T were amplified by polymerase chain reaction (PCR) and sequenced. *PTGDR* expression levels were analyzed using quantitative PCR and normalized to *GAPDH* and *TBP* mRNA levels. All procedures were performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiment guidelines.
Results: PTGDR expression levels were significantly higher in allergic patients than in controls (*P*<.001). Receiver operating characteristic analysis for expression of *PTGDR* showed a sensitivity of 81.4% compared with 67% for IgE levels. In addition, differences in the genotypic distribution of the polymorphisms –1289G>A and –1122T>C were found in allergic patients (*P*=.009).
Conclusions: The results indicate that *PTGDR* overexpression is associated with allergy. The polymorphisms –1289G>A and –1122T>C partly explain the variation in expression we observed. *PTGDR* expression could have a potential role as a biomarker and pharmacogenetic factor in allergy.

Key words: Allergy. Asthma. mRNA expression. Polymorphisms. *PTGDR*. Rhinitis.

Resumen

Antecedentes: Los receptores de la prostaglandina D2 están adquiriendo un papel relevante como posibles dianas terapéuticas en la alergia. El gen *PTGDR* ha sido descrito como un gen candidato en una enfermedad alérgica, sin embargo, faltan estudios funcionales sobre este gen.
Objetivo: El objetivo de este estudio de casos y controles fue analizar el posible papel del gen *PTGDR* en la alergia.
Métodos: Se incluyeron 195 pacientes alérgicos y 112 controles sanos. Un fragmento de la región promotora de *PTGDR* que comprendía las posiciones polimórficas -1289G> A, -1122T>C, -881C>T, -834C>T, -613C>T, -549T>C, -441C>T, -197T>C y -95G>T fue amplificado mediante la reacción en cadena de la polimerasa y secuenciado. Los niveles de expresión de *PTGDR* se analizaron mediante q-PCR y se normalizaron a los niveles de ARNm de *GAPDH* y *TBP*. Todos los procedimientos se realizaron siguiendo la guía MIQE.
Resultados: Los niveles de expresión de *PTGDR* fueron significativamente superiores en los pacientes alérgicos que en los controles (p<0,001). El análisis ROC para la expresión de *PTGDR* mostró una sensibilidad del 81,4% en comparación con el 67% para los niveles de IgE. Además, se encontraron diferencias en la distribución genotípica de los polimorfismos -1289G>A y -1122T>C en pacientes alérgicos (p=0,009).
Conclusiones: Los resultados indican que la sobreexpresión de *PTGDR* se asocia con la alergia. Además, los polimorfismos -1289G>A y -1122T>C contribuyen a explicar parte de la variación de expresión observada. La expresión de *PTGDR* podría tener un papel potencial como biomarcador y factor farmacogenético en la alergia.

Palabras clave: Alergia. Asma. Expresión génica. Polimorfismos. *PTGDR*. Rinitis.

Introduction

Allergy does not follow a classical Mendelian inheritance pattern, which is typical of monogenic disorders, but a multifactorial pattern, which involves interactions between different genetic and environmental factors [1]. Genome-wide association studies (GWASs) performed in the field of allergy have indicated that the disease can be influenced by a large number of genes [2]. The prostaglandin D2 receptor gene, *PTGDR*, has been proposed as a candidate gene in asthma (including allergic asthma) [3,4]. Prostaglandin D2 (PGD2) is increased after exposure to allergens [5]. The production of PGD2 has been linked to allergic asthma, atopic dermatitis, allergic rhinitis, and conjunctivitis, and PGD2 has been detected in the skin, tears, and nasal secretions of allergic patients [6]. Locally, PGD2 acts through 2 transmembrane receptors, PTGDR (prostaglandin D2 receptor or DP1) and CRTH2 (chemokine receptor-homologous molecule expressed on T_H2 lymphocytes or DP2), which belong to the superfamily of heterotrimeric G-protein-coupled receptors [7].

Linkage studies showed that a region located on chromosome 14 of *PTGDR* was a candidate for hosting genetic markers linked to asthma and related phenotypes [8,9]. Association studies revealed an association between *PTGDR* and allergic diseases [2,3,10,11]. Nevertheless, replication of association studies on *PTGDR* is controversial with respect to ethnicity and other factors. Thus, single-nucleotide polymorphisms (SNPs) of *PTGDR* have been associated with asthma in Caucasian populations [3,4] and in an African American population [4], but not in Australian [12], Mexican, Puerto Rican, another African American [13], or Chinese Han populations [14]. In addition, chronic rhinosinusitis with nasal polyposis has been associated with various prostaglandin receptors, including *PTGDR*, and with prostaglandin D2 synthase (PTGDS) [15]. Despite ethnic differences, *PTGDR* is one of the genes whose association with asthma and atopic asthma has been replicated [16], specifically in the case of polymorphisms of the promoter region, such as –549C/T, –441C/T, and –197C/T [3,4,10]. These polymorphisms are located at binding sites of transcription factors, which could modify transcription levels [4]. Some haplotypic combinations of these variants have been associated with increased transcriptional activity of *PTGDR* [3,4,10,11].

In this study, we investigated the role of *PTGDR* in allergic diseases, including allergic asthma. Our objectives were as follows: firstly, to compare *PTGDR* expression levels in allergic patients and controls; secondly, to compare allelic, genotypic, and haplotypic frequencies of 9 *PTGDR* promoter polymorphisms in these 2 populations; and lastly, to examine the putative relationship between *PTGDR* expression levels and the presence of *PTGDR* promoter polymorphisms.

Methods

Study Population

For this observational, analytical, case-control study of 314 white individuals (age >16 years), potentially eligible participants were selected over a period of 4 years, with 307 finally included. Seven samples were excluded because they did not fulfill the quality criteria for molecular analysis. The statistical power of the sample size was analyzed. The study was approved by the Ethics Committee of University Hospital of Salamanca, Salamanca, Spain, and participants gave their written informed consent. A total of 112 individuals were enrolled as controls, provided they met the following criteria: (*1*) no symptoms or history of asthma or other pulmonary diseases; (*2*) no symptoms or history of rhinitis; (*3*) no symptoms or history of allergic diseases; (*4*) negative results in skin prick tests with a battery of common aeroallergens; (*5*) absence of family history of asthma, rhinitis, or atopy. The 195 patients recruited fulfilled the following criteria: (*1*) physician-based diagnosis of respiratory allergy (allergic asthma and/or allergic rhinitis); (*2*) at least 1 positive skin prick test to a battery of previously described common aeroallergens [3]. Patients were considered to have allergic asthma if they had asthmatic symptoms related to exposure to the aeroallergens they were sensitized to and a positive bronchodilator or methacholine test result. Patients were diagnosed with allergic rhinitis if symptoms suggesting allergic rhinitis were present and correlated with patients' sensitizations. Skin prick tests were performed following the recommendations of the European Academy of Allergy and Clinical Immunology (EAACI) allergen standardization and skin test subcommittee [17]. Skin tests were considered positive if there was at least 1 wheal reaction of >3 mm in diameter greater than the negative control. Patients had to have a positive skin prick test result to at least 1 allergen. Patients were considered monosensitized if they had a positive skin prick test result to only 1 group of aeroallergens (house dust mites, molds, pollens, or animal dander) and polysensitized if they had positive skin test results to 2 or more groups.

Total serum IgE levels were measured using a fluoroenzyme immunoassay (Thermo Fisher Scientific).

Genotype Analysis

DNA was extracted from whole blood using the automated MagNA Pure Compact System (Roche Applied Science). A 1416-bp fragment comprising the 9 SNP positions of the *PTGDR* promoter region (–1289G>A, –1122T>C, –881C>T, –834C>T, –613C>T, –549T>C, –441C>T, –197T>C, and –95G>T) was amplified by polymerase chain reaction (PCR). The forward and reverse primers used in these amplifications were 5' GGCATGAGGCCTAAAAATGAG 3' and 5' GAAGAAGGCGAAGGCTTGG 3', respectively. Reaction mixtures and cycling conditions were as previously described [10]. ExoSAP-IT (USB) was used to purify amplified fragments. Amplicons were sequenced in an ABI PRISM 377 DNA Sequencer (Thermo Fisher Scientific). Chromas 2.3 (Technelysium) was used for the analysis. Specific quality measures were taken in all procedures following European Molecular Genetics Quality Network guidelines [18].

RNA Extraction and cDNA Preparation

Total RNA was isolated from peripheral blood using the RiboPure-Blood kit (Thermo Fisher Scientific). DNase was treated using Turbo DNAse (Thermo Fisher Scientific).

RNA was quantified in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The RNA integrity number (RIN) algorithm was used to determine the quality of RNA in an Agilent 2100 Bioanalyzer (Agilent Technologies). In order to ensure the viability of gene expression experiments, only RNA samples with a RIN value above 7 were used. cDNA was generated using the Superscript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific). Samples were stored at –20ºC until use.

Selection of Constitutive Expression Genes

Constitutive genes were selected using the Real-Time ready Human Reference Gene Panel (Roche Applied Science). On each plate, samples from patients and controls were added in duplicate to obtain the cycle threshold (Ct) of all housekeeping genes. The mean (SD) Ct was calculated for controls and patients for each of the 19 housekeeping genes in order to evaluate its variability. The mean Ct of housekeeping genes was estimated in controls and patients by determining the coefficient of variation from the total Ct average. Finally, analysis of variance tests were performed after verifying normality with the Kolmogorov-Smirnov test and homogeneity of variances with the Levene test.

Primer Efficiency Study

PTGDR primers were designed using the Primer 3Plus software package. An 82-bp *PTGDR* fragment was amplified with the forward and reverse primers 5'- GGCATGAGGCCTAAAAATGAG-3' and 5'-CCTTGACATCCTTAAATGCTCC-3', respectively. Primer sequences were verified to ensure that they did not contain any known polymorphism in order to avoid variations in specificity. The efficiency study was carried out for *PTGDR* qPCR primers, as well as for *GAPDH* and *TBP* primers of the Real-Time ready Human Reference Gene Panel (Roche Applied Science) [19]. The *GAPDH* forward and reverse primers were 5'-CTCTGCTCCTCCTGTTCGAC-3' and 5'-ACGACCAAATCCGTTGACTC-3', respectively. The *TBP* forward and reverse primers were 5'-GAACATCATGGATCAGAACAACA-3' and 5'-ATAGGGATTCCGGGAGTCAT-3', respectively. Serial dilutions of a cDNA sample of a known concentration were used, and efficiency was calculated according to the equation $E = 10^{[-1/slope]}$ [20] in the cDNA concentration range of 0.39 to 100 ng, with high linearity (Pearson correlation coefficient >0.95) [21].

Real-time Quantitative PCR

Expression analyses of the 3 genes were performed in a LightCycler 480 system (Roche Applied Science). Each well contained a final PCR reaction volume of 15 µL based on 7.5 µL Master Mix SYBR Green I (2X) (Roche Applied Science), 600 nM of each forward and reverse primer, and 25 ng of cDNA template. The PCR conditions included 10 minutes at 95ºC followed by 45 cycles of 10 seconds at 95ºC for denaturation, 10 seconds at 60ºC for annealing, and 10 seconds at 72ºC for polymerization. Finally, melting curve analyses were conducted to check the specificity of the qPCR products. All reactions were performed in triplicate, and nontemplate controls were included in each experiment to detect contamination. In order to detect and correct potential interassay variations, a known concentration sample (calibrator) was included. Control and patient samples were included in each experiment. The Ct was automatically determined using the LightCycler 480 software. *PTGDR* expression levels were normalized to *GAPDH* and *TBP* mRNA levels using the Livak Method [22]. All procedures were performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiment (MIQE) guidelines [23]. Expression was not analyzed in 15 samples that did not have a RIN value >7 (8 controls, 7 patients).

Statistical Analysis

Qualitative variables were analyzed using the χ^2, Fisher exact, and Monte Carlo (10^4 simulations) tests; continuous variables were analyzed across each genotype using the ANOVA, Kruskal-Wallis, and Mann-Whitney tests. Homoscedasticity was analyzed accordingly. Logistic regression was used to model the effects of multiple covariates. Age and sex were included as potential covariates in the multivariate analysis. Analyses of allelic and genotypic distributions, as well as a study of haplotype distribution and the Hardy-Weinberg equilibrium, were performed using the SHEsis online platform [24]. Correction for multiple comparisons, false-positive report probability, and statistical power were also assessed as a statistical control. Statistical analyses were performed using SPSS 17.0 software (SPSS Inc.). Receiver operating curve analysis was applied for the sensitivity and specificity study.

Results

Characteristics of the Study Population

The phenotypic characteristics of the study population are shown in Table 1. Briefly, age was significantly lower in patients than in controls ($P<.001$), and total IgE levels were significantly lower in controls ($P<.001$). In addition, patients diagnosed with both allergic asthma and allergic rhinitis had significantly higher IgE levels than patients with rhinitis only ($P=.013$).

The severity of asthma was evaluated according to GINA guidelines [25]. Intermittent asthma was the most common type (48%), followed by moderate persistent asthma (32%). Rhinitis was classified following the ARIA guidelines, as modified by Valero et al [26], with moderate persistent rhinitis (41%) and moderate intermittent rhinitis (22%) being the most frequent types. The most common aeroallergen sensitization was to pollen (146 patients), followed by mites (139 patients).

Selection of Reference Genes

A general recommendation in gene expression studies is to choose reference genes with a similar expression level to that of the target gene [27]. Consequently, and given that it has the lowest variability among all of the genes analyzed, *TBP* was selected as the reference gene (see Table 1, Supplementary

Table 1. Characteristics of the Study Population

	Population	Controls	Allergic Patients	Patients With Allergic Asthma	Patients With Allergic Rhinitis	Patients With Allergic Rhinitis Without Asthma
Patients	307	112	195	137	189	58
Sex, %						
Female	53.4	57.1	51.3	51.8	50.3	50
Male	46.6	42.9	48.7	48.2	49.7	50
Mean (SD) age, y	41.5 (19.1)	56.7 (17.4)	32.9 (14.1)	32.7 (13.9)	32.5 (13.6)	33.5 (14.5)
Mean (SD) IgE, kU/L	269.6 (382.1)	99.6 (178.5)	361.5 (428.9)	407.7 (460.5)	365.9 (432.1)	258.1 (327.9)
P value[a]			*P*<.001	*P*<.001	*P*<.001	*P*<.001
P value[b]				*P*=.013		*P*=.010

[a]*P* value for each group of patients vs controls
[b]*P* value for allergic patients without asthma vs patients with allergic asthma; patients with allergic rhinitis and asthma vs patients with allergic rhinitis only.

material). Following MIQE guidelines, 2 housekeeping genes should be included. In this sense, *GAPDH* was also included because it has been broadly used in gene expression studies of asthma [28,29]. Before evaluating expression data, the correlation between the 2 selected reference genes was confirmed (Spearman ρ, 0.783; *P*<.001).

Expression Analysis

PTGDR expression levels were significantly higher in allergic patients than in controls (*P*<.001). This highly significant difference was also observed in all subgroups of allergic patients. No significant differences in *PTGDR* expression levels were observed between the different subgroups of allergic patients, although *PTGDR* expression levels were slightly higher in patients sensitized to pollens (Table 2).

We investigated the possible influence of anti-inflammatory medications on the expression of *PTGDR*. No patients were taking nonsteroidal oral anti-inflammatory drugs or oral corticosteroids at blood sampling. In addition, 32 patients were receiving allergen immunotherapy (AIT), and a further 19 patients had previously received AIT. No statistically significant differences (*P*=.436) in the expression levels of

Table 2. *PTGDR* Expression Levels According to Diagnosis and Sensitization

	No.	Mean (SD) Ct	*P* Value[a]	Statistical Power
Controls	104	0.68 (0.52)		
Allergic patients	188	0.92 (0.52)	<.001	95.4%
Allergy to mites (at least)	133	0.87 (0.49)	<.001	80.6%
Allergy to mites (only)	37	0.94 (0.63)	.016	72.2%
Allergy to pollens (at least)	140	0.92 (0.51)	<.001	94.7%
Allergy to pollens (only)	34	0.98 (0.42)	.03	96.2%
Allergic asthma	131	0.92 (0.55)	<.001	92.2%
Allergic asthma (polysensitized)	89	0.90 (0.54)	<.001	81.3%
Allergic asthma (monosensitized to mites)	21	0.95 (0.70)	.044	38.6%
Allergic asthma (monosensitized to pollens)	21	0.96 (0.43)	.003	75.1%
Allergic rhinitis	182	0.91 (0.50)	<.001	94.4%
Allergic rhinitis (polysensitized)	114	0.89 (0.52)	<.001	84.9%
Allergic rhinitis (monosensitized to mites)	34	0.87 (0.50)	.016	46.5%
Allergic rhinitis (monosensitized to pollens)	34	0.98 (0.42)	<.001	92.8%

Abbreviation: Ct, cycle threshold.
[a]For each group of patients, the *P* value results from comparing the expression values in that group of patients with the values of the control group.

Table 3. Comparison Between *PTGDR* mRNA Expression Levels and Peripheral Blood Total IgE Levels

	PTGDR (Mean Ct)	IgE, kU/L
Cut-off	0.509	100 kU/L
Sensitivity	81.4%	67%
Specificity	42.3%	75%
Positive predictive value	72%	83%
Negative predictive value	55%	55%

Abbreviation: Ct, cycle threshold.

PTGDR were observed in patients receiving AIT with respect to patients not receiving AIT. Concerning pollen allergy, the sample was obtained during the pollen season in 58 of 152 patients. Again, no statistically significant differences were observed when the sample was obtained during the pollen season or outside it (*P*=.624). This also implies that the medication used to treat pollen allergy, in particular nasal and/or inhaled corticosteroids, had no effect on the expression of *PTGDR*. In addition, the bioavailability of inhaled corticosteroids, which are administered at higher doses than nasal corticosteroids, is very low, and even negligible, in some cases [30]. Exposure to the remaining aeroallergens was not seasonal.

PTGDR Expression Levels as a Potential Biomarker of Allergy Sensitization

We compared *PTGDR* mRNA expression levels with serum IgE levels as biomarkers of allergy. Receiver operating characteristic curve analysis revealed an area under the curve of 0.654 (95%CI, 0.588-0.721), thus providing a cut-off for *PTGDR* expression of 0.509 (Table 3).

Total IgE levels increased significantly according to the number of sensitization groups; however, these differences were not observed for *PTGDR* expression levels (Table 4).

Table 4. Total IgE and *PTGDR* Expression Levels According to the Number of Sensitization Groups in Patients

No. of Sensitizations[a]	Total IgE, kU/L Mean (SD)	*PTGDR* Mean (SD) Ct
1 (n=71)	246.3 (313.6)	0.960 (0.533)
2 (n=58)	363.2 (446.1)	0.889 (0.572)
3 or more (n=59)	490.8 (490.6)	0.885 (0.461)
P value	[b].005	[c].652

Abbreviation: Ct, cycle threshold.
[a]Number of sensitizations refers to the number of groups of aeroallergens (pollens, mites, epithelia, and molds) to which patients were sensitized.
[b]*P* value obtained for the comparison of IgE levels among patients sensitized to 1, 2, 3, or 4 groups of allergens.
[c]*P* value obtained for the comparison of *PTGDR* expression levels among patients sensitized to 1, 2, 3, or 4 groups of allergens.

Genetic Association Analysis

In the association study of the 9 *PTGDR* promoter polymorphisms, statistically significant differences between allergic patients and controls were observed for the allelic and the genotypic frequencies of –1289G>A and –1122T>C. As was the case with *PTGDR* expression, the differences in the genotype distribution of these SNPs between allergic patients and controls were also observed for the subphenotypes of atopic asthma and atopic rhinitis. When the type of allergic sensitization was taken into account, significant differences were observed in patients with allergy to pollens (*P*=.028). Furthermore, in the group of patients sensitized to mites and pollens, statistically significant differences were found for –1289G>A and –1122T>C after adjusting for sex and age (*P*=.009 [OR, 0.24; 95%CI, 0.08-0.70]; false-positive report probability = 14.4%). The linkage study carried out for –1289G>A and –1122T>C yielded a coefficient of linkage disequilibrium of 1 and an r^2 of 0.985. The Hardy-Weinberg equilibrium was fulfilled in all cases, except in the case of 441C>T in the control group (Table 5). No statistically significant differences were observed for the remaining SNPs between patients and controls (Table 5) or for the general haplotype distribution (Table 6).

Association Between PTGDR Promoter Polymorphisms and Expression Levels

In the total population of patients and controls, the mutant homozygous genotypes AA and CC of polymorphisms –1289G>A and –1122T>C, respectively, were both associated with statistically significantly lower *PTGDR* expression levels (*P*=.034) (Table 7). These differences were confirmed in the subgroup of patients simultaneously sensitized to pollen and mites, where genotypes carrying the mutant alleles are associated with significantly lower *PTGDR* expression (*P*=.009). Interestingly, the mutant alleles associated with lower *PTGDR* levels were significantly more frequent in controls than in any of the allergic groups (Table 7).

Discussion

In the present study, highly significant differences were observed in *PTGDR* expression levels between controls and allergic patients. Thus, *PTGDR* expression levels were higher in allergic patients than in nonallergic participants. *PTGDR* expression levels were independent of aeroallergen sensitization group and/or clinical manifestations, although expression levels were slightly higher in patients sensitized to pollens, suggesting that expression levels of *PTGDR* could be a general trait of atopy. In this sense, arachidonic acid derivatives such as prostaglandins and leukotrienes are important mediators in allergic reactions and are responsible for many of the symptoms of rhinitis and asthma. Thus, PGD2 is the cyclooxygenase metabolite of arachidonic acid that is most abundantly produced in response to environmental allergens and has been proposed as a mast cell activation marker [31]. PGD2 produces bronchoconstriction, vasodilation, and increased capillary permeability and mucus production in allergic patients [32].

Table 5. Genotypic and Allelic Frequencies of *PTGDR* Promoter SNPs

Phenotype		Genotype				Allele			
–1289G>A	No.	GG	GA	AA	*P* Value[a]	G	A	*P* Value	OR (95%CI)
Controls	112	0.38	0.52	0.10		0.64	0.36		
All allergic patients	195	0.53	0.39	0.08	.044/.045	0.73	0.27	.027	0.67 (0.47-0.96)
Allergic asthma[b]	136	0.54	0.40	0.06	.045/.062	0.74	0.26	.019	0.63 (0.43-0.92)
Allergic rhinitis[c]	188	0.53	0.39	0.08	.054/.047	0.72	0.28	.035	0.68 (0.48-0.97)
Pollen allergy (at least)[d]	145	0.56	0.37	0.08	.019/.028	0.74	0.26	.014	0.62 (0.42-0.91)
Mite + pollen allergy (at least)[e]	90	0.57	0.33	0.10	.023/.031	0.73	0.27	.048	0.65 (0.42-0.99)
Mite + pollen allergy (only)[f]	32	0.63	0.31	0.06	.050/.009	0.78	0.22	.035	0.50 (0.26-0.96)
–1122T>C	No.	TT	TC	CC	*P* Value[a]	T	C	*P* Value	OR (95%CI)
Controls	112	0.38	0.52	0.10		0.64	0.36		
All allergic patients	195	0.53	0.39	0.08	.053/.047	0.72	0.28	.038	0.69 (0.48-0.98)
Allergic asthma	136	0.53	0.40	0.07	.066/.064	0.73	0.27	.030	0.65 (0.45-0.96)
Allergic rhinitis	188	0.52	0.39	0.09	.064/.048	0.72	0.28	.049	0.70 (0.49-0.99)
Pollen allergy (at least)	145	0.55	0.37	0.08	.025/.029	0.73	0.27	.023	0.64 (0.44-0.94)
Mite + pollen allergy (at least)	90	0.57	0.33	0.10	.023/.031	0.73	0.27	.048	0.65 (0.42-0.99)
Mite + pollen allergy (only)	32	0.63	0.31	0.06	.050/.009	0.78	0.22	.035	0.50 (0.26-0.96)
–881C>T	No.	CC	CT	TT	*P* Value	C	T	*P* Value	OR (95%CI)
Controls	112	0.80	0.20	0.00		0.90	0.10		
All allergic patients	195	0.78	0.21	0.01	.544	0.88	0.12	.545	0.85 (0.49-1.45)
Allergic asthma	136	0.75	0.23	0.02	.372	0.87	0.13	.323	0.75 (0.43-1.32)
Allergic rhinitis	188	0.78	0.21	0.01	.529	0.88	0.12	.523	0.84 (0.49-1.44)
Pollen allergy (at least)	145	0.76	0.23	0.01	.392	0.87	0.13	.335	0.76 (0.43/1.33)
Mite + pollen allergy (at least)	90	0.80	0.18	0.02	.276	0.89	0.11	.718	0.89 (0.47-1.69)
Mite + pollen allergy (only)	32	0.69	0.28	0.03	.101	0.83	0.17	.114	0.53 (0.24-1.17)
–834C>T	No.	CC	CT	TT	*P* Value	C	T	*P* Value	OR (95%CI)
Controls	112	0.59	0.35	0.06		0.76	0.24		
All allergic patients	195	0.50	0.42	0.08	.354	0.71	0.29	.182	0.77 (0.53-1.13)
Allergic asthma	136	0.51	0.43	0.06	.416	0.72	0.28	.351	0.82 (0.55-1.24)
Allergic rhinitis	188	0.51	0.42	0.07	.405	0.72	0.28	.221	0.79 (0.54-1.15)
Pollen allergy (at least)	145	0.49	0.44	0.07	.303	0.71	0.29	.197	0.77 (0.52-1.15)
Mite + pollen allergy (at least)	90	0.49	0.43	0.08	.392	0.71	0.29	.208	0.75 (0.48-1.17)
Mite + pollen allergy (only)	32	0.50	0.41	0.09	.650	0.70	0.30	.345	0.74 (0.40-1.38)
–613C>T	No.	CC	CT	TT	*P* Value	C	T	*P* Value	OR (95%CI)
Controls	112	0.79	0.21	0.00		0.90	0.10		
All allergic patients	195	0.78	0.21	0.01	.557	0.88	0.12	.640	0.88 (0.51-1.50)
Allergic asthma	136	0.75	0.23	0.02	.396	0.87	0.13	.389	0.78 (0.45-1.37)
Allergic rhinitis	188	0.78	0.21	0.01	.545	0.88	0.12	.615	0.87 (0.51-1.49)
Pollen allergy (at least)	145	0.76	0.23	0.01	.418	0.87	0.13	.403	0.79 (0.45-1.37)
Mite + pollen allergy (at least)	90	0.80	0.18	0.02	.260	0.89	0.11	.809	0.92 (0.49-1.74)
Mite + pollen allergy (only)	32	0.69	0.28	0.03	.109	0.83	0.17	.137	0.56 (0.25-1.21)

Continued

Table 5. Genotypic and Allelic Frequencies of *PTGDR* Promoter SNPs (*Continuation*)

Phenotype		Genotype				Allele			
–549T>C	No.	CC	CT	TT	*P* Value	C	T	*P* Value	OR (95%CI)
Controls	112	0.36	0.46	0.18		0.59	0.41		
All allergic patients	195	0.27	0.51	0.22	.235	0.53	0.47	.125	0.77 (0.55-1.07)
Allergic asthma	136	0.27	0.52	0.21	.329	0.53	0.47	.204	0.79 (0.55-1.13)
Allergic rhinitis	188	0.27	0.52	0.21	.270	0.53	0.47	.167	0.79 (0.56-1.10)
Pollen allergy (at least)	145	0.24	0.55	0.21	.117	0.51	0.49	.086	0.73 (0.52-1.04)
Mite + pollen allergy (at least)	90	0.28	0.51	0.21	.457	0.53	0.47	.254	0.79 (0.53-1.18)
Mite + pollen allergy (only)	32	0.31	0.47	0.22	.834	0.55	0.45	.537	0.84 (0.48-1.47)
–441C>T	No.	CC	CT	TT	*P* Value	C	T	*P* Value	OR (95%CI)
Controls	112	0.70	0.23	0.07		0.82	0.18		
All allergic patients	195	0.70	0.25	0.05	.874	0.82	0.18	.942	1.01 (0.66-1.56)
Allergic asthma	136	0.67	0.29	0.04	.456	0.82	0.18	.999	1.00 (0.63-1.58)
Allergic rhinitis	188	0.70	0.25	0.05	.807	0.82	0.18	.886	1.03 (0.67-1.59)
Pollen allergy (at least)	145	0.68	0.27	0.05	.691	0.81	0.19	.861	0.96 (0.61-1.51)
Mite + pollen allergy (at least)	90	0.70	0.27	0.03	.579	0.83	0.17	.722	1.09 (0.65-1.85)
Mite + pollen allergy (only)	32	0.75	0.19	0.06	.852	0.84	0.16	.657	1.19 (0.56-2.53)
–197T>C	No.	TT	TC	CC	*P* Value	T	C	*P* Value	OR (95%CI)
Controls	112	0.81	0.18	0.01		0.90	0.10		
All allergic patients	195	0.77	0.21	0.02	.707	0.88	0.12	.408	1.25 (0.73-2.14)
Allergic asthma	136	0.77	0.21	0.02	.617	0.87	0.13	.366	1.30 (0.73-2.29)
Allergic rhinitis	188	0.77	0.21	0.02	.684	0.88	0.12	.387	1.27 (0.74-2.17)
Pollen allergy (at least)	145	0.79	0.20	0.01	.861	0.89	0.11	.594	1.17 (0.66-2.06)
Mite + pollen allergy (at least)	90	0.72	0.27	0.01	.329	0.86	0.14	.163	1.53 (0.84-2.81)
Mite + pollen allergy (only)	32	0.72	0.28	0.00	.405	0.86	0.14	.346	1.49 (0.65-3.41)
–95G>T	No.	GG	GT	TT	*P* Value	G	T	*P* Value	OR (95%CI)
Controls	112	0.99	0.01	0.00		0.99	0.01		
All allergic patients	195	0.97	0.03	0.00	.310	0.97	0.03	.310	0.34 (0.03-2.97)
Allergic asthma	136	0.98	0.02	0.00	.419	0.99	0.01	.421	0.41 (0.04-3.93)
Allergic rhinitis	188	0.97	0.03	0.00	.295	0.99	0.01	.297	0.34 (0.04-2.89)
Pollen allergy (at least)	145	0.99	0.01	0.00	.724	0.99	0.01	.775	0.65 (0.06-7.23)
Mite + pollen allergy (at least)	90	0.98	0.02	0.00	.442	0.99	0.01	.444	0.40 (0.03-4.48)
Mite + pollen allergy (only)	32	0.97	0.03	0.00	.345	0.98	0.02	.347	0.28 (0.02-4.62)

[a]*P* value: Fisher *P* value/logistic regression *P* value adjusted for age and sex covariates. For each group of patients, the *P* values and OR were obtained by comparing the genotypic and allelic distributions in that group of patients with those of the control group.
[b]Allergic asthma: most of these patients also had allergic rhinitis.
[c]Allergic rhinitis: two-thirds had concomitant asthma.
[d]Pollen allergy (at least): Patients with allergy to at least pollens (most of them may have other sensitizations).
[e]Mite + pollen (at least): Patients with allergy to at least pollens and mites (some of them may have other sensitizations).
[f]Mite + pollen allergy (only): Patients with allergy to pollens and mites (but not to any other group of aeroallergens).

Table 6. Haplotypic Frequencies[a]

Haplotype	Control	Allergic Patients	Allergic Asthma	Allergic Rhinitis	Pollen Allergy (at Least)	Mite + Pollen Allergy (at Least)	Mite + Pollen Allergy (Only)
ACCCCCCTG	0.36	0.27 *P*=.017	0.25 *P*=.013	0.27 *P*=.023	0.25 *P*=.009	0.26 *P*=.027	0.22 *P*=.032
GTCCCCCCG	0.10	0.11 *P*=.673	0.11 *P*=.485	0.11 *P*=.635	0.10 *P*=.728	0.13 *P*=.302	0.13 *P*=.507
GTCCCCCCT	0.00	0.01 *P*=.404	0.0 *P*=.495	0.01 *P*=.384	0.01 -	0.01 *P*=.475	0.02 *P*=.259
GTCCCCCTG	0.03	0.02 *P*=.400	0.02 *P*=.513	0.02 *P*=.438	0.02 *P*=.447	0.02 *P*=.563	0.02 *P*=.485
GTCCCTTTG	0.17	0.18 *P*=.735	0.18 *P*=.691	0.18 *P*=.789	0.19 *P*=.546	0.17 *P*=.976	0.16 *P*=.799
GTCTCTCTG	0.23	0.29 *P*=.117	0.28 *P*=.241	0.29 *P*=.144	0.29 *P*=.117	0.29 *P*=.151	0.30 *P*=.306
GTTCTCCTG	0.10	0.11 *P*=.591	0.13 *P*=.365	0.11 *P*=.568	0.12 *P*=.364	0.11 *P*=.713	0.17 *P*=.121

[a]Data represent the percentage of each haplotype in each phenotypic group and the corresponding Fisher *P* value. Haplotypes with a frequency >1% among either controls or patients are included. The order of the single-nucleotide polymorphisms in each haplotype is –1289G>A, –1122T>C, –881C>T, –834C<T, –613C>T, –549T>C, –441C>T, –197T>C, and –95G>T. Please see the legend of Table 5.

Table 7. *PTGDR* Expression Levels According to the –1289G>A and –1122T>C Genotypes[a]

		–1289G>A				–1122T>C			
		GG	GA	AA	*P* Value	TT	TC	CC	*P* Value
Controls	Frequency	0.38	0.52	0.10		0.38	0.52	0.10	
	Mean (SD) Ct	0.76 (0.62)	0.67 (0.47)	0.43 (0.27)	.160	0.75 (0.62)	0.67 (0.46)	0.42 (0.26)	.161
Allergic patients	Frequency	0.53	0.39	0.08	.045	0.53	0.39	0.08	.045
	Mean (SD) Ct	0.93 (0.47)	0.91 (0.59)	0.79 (0.51)	.580	0.93 (0.47)	0.91 (0.59)	0.79 (0.48)	.600
Pollen allergy (at least)	Frequency	0.56	0.37	0.07	.028	0.55	0.37	0.08	.029
	Mean (SD) Ct	0.93 (0.41)	0.94 (0.62)	0.79 (0.52)	.540	0.93 (0.42)	0.94 (0.62)	0.79 (0.49)	.541
Pollen + mite allergy (at least)	Frequency	0.57	0.33	0.10	.031	0.57	0.33	0.10	.031
	Mean (SD) Ct	0.92 (0.44)	0.75 (0.42)	0.87 (0.55)	.340	0.92 (0.44)	0.75 (0.42)	0.87 (0.55)	.340
Pollen + mite allergy (only)	Frequency	0.63	0.31	0.06	.009	0.63	0.31	0.06	.009
	Mean (SD) Ct	1.06 (0.54)	0.48 (0.41)	0.58 (0.32)	.008	1.06 (0.54)	0.48 (0.41)	0.58 (0.32)	.010

Abbreviation: Ct, cycle threshold.
[a]Pollen + mite allergy (at least) group includes patients with allergy to both pollens and mites regardless of whether they could have other sensitizations. The group pollen + mite allergy (only) includes patients who only have allergy to pollen and mites but not to any other group of aeroallergens.

Expression studies require rigorous quality controls with respect to sample collection and characterization, qPCR primers, nucleic acid extraction, RT-PCR, target gene information, the qPCR protocol, qPCR validation, and analysis of data [23]. Therefore, such quality controls should be followed in order to provide reliable results. In this sense, we carefully followed all MIQE recommendations and performed a previous in-depth analysis in order to select the most appropriate constitutive genes from 19 candidates. We were able to clearly differentiate between allergic patients and controls based on expression of *PTGDR*. In addition, the gene can be detected by a simple and rapid technique from an easily obtained biological sample and has better sensitivity than IgE levels. However, its low specificity and negative predictive value limit its potential for differential diagnosis. We found that total IgE levels increased according to the number of sensitization groups [33]. This was not the case for *PTGDR* levels, which did not exhibit significant differences regarding the number of sensitization groups. Consequently, *PTGDR* could be a good indicator of the inflammation activation

mechanism of allergy, rather than of severity. In addition, our results show that *PTGDR* expression could be considered a complementary diagnostic test when it is suspected that IgE determination is not sufficiently sensitive. With regard to the genotyping analysis, we found that the mutant alleles of the SNPs –1289G>A and –1122T>C were significantly more frequent in controls than in any of the groups of allergic patients. Furthermore, these mutant alleles were associated with lower levels of *PTGDR* expression, thus suggesting that the presence of these SNPs could be associated with a decreased risk of developing the disease. Both SNPs were in linkage disequilibrium, suggesting that the analysis of only one of them could be sufficiently informative.

Some of the polymorphisms analyzed in this study have previously been associated with asthma. Thus, the –549T>C variant has been associated with asthma in whites and African Americans, and the –441T>C variant has been associated with asthma in a white population [4]. In a Spanish population, the SNP –197T>C was associated with asthma, particularly allergic asthma [11]. In another study, the SNP –613C>T was associated with allergy, mainly with cosensitization to mites and pollens, whereas the differences detected for the SNP –549T>C were almost significant (*P*=.09) in patients sensitized to both pollen and mites [11]. However, studies in Mexican, Puerto Rican, and African American populations showed no involvement of–549T>C, –441T>C, or –197T> C in the development of asthma [13]. Similar findings have been reported for an Australian population [12]. These discrepancies may be due to small ethnic differences between populations or to environmental factors, since these polymorphisms were only associated with asthma in white populations. We found no significant association for these SNPs, although it should be taken into account that that our study focused on allergic patients and not on asthma. Most previous studies lack a functional analysis. In our study, the results of the expression analysis suggest that the association of both –1289G>A and –1122T>C could be related to their transcriptional role. We emphasized that expression analysis can help to identify the mechanisms underlying the inflammatory pathways in allergic disease and to verify the functional role of suggested SNPs in association studies. We showed that expression of *PTGDR* seems to be determined in part by the presence of the SNPs –1289G>A and –1122T>C. However, for the same genotype, expression levels still differed between controls and allergic patients, thus indicating that additional factors such as other SNPs and/or epigenetic factors affect *PTGDR* expression.

Our findings are potentially limited by the study sample size, although this was offset by the statistical power. In addition, the sex distribution between the patient and control groups was resolved by logistic regression. The probability of a type 1 error was resolved by applying the Bonferroni correction and the false-positive report probability. Finally, the putative selection of an inappropriate constitutive gene was resolved by analyzing 19 genes in both the controls and the patients.

Our study provides interesting insights into *PTGDR* as a potential predictive marker of allergy. Examination of this role could in turn provide more data on its role in disease and in activation of the inflammation pathway. In addition, our findings could contribute to the analysis of PGD2 receptors as therapeutic targets by highlighting the potential role of genetic variants that impair expression of PGD2 receptors as possible pharmacogenetic predictors of therapeutic response to their antagonists [34].

Further studies are required to validate our results in different populations and could reveal specific mechanisms by which increased expression of *PTGDR* is related to the occurrence of allergic phenotypes and how it can impact on therapeutic strategies.

Conclusions

PTGDR expression levels were associated with allergy. The promoter SNPs –1289G>A and –1122T>C could be responsible for some of the variation in the expression levels observed in patients. *PTGDR* could play a role as biomarker and pharmacogenetic factor in allergy.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

1. Holloway JW, Yang IA, Holgate ST. Genetics of allergic disease. J Allergy Clin Immunol. 2010;125(2):81-94.
2. Portelli MA, Hodge E, Sayers I. Genetic risk factors for the development of allergic disease identified by genome-wide association. Clin Exp Allergy. 2015;45(1):21-31.
3. Sanz C, Isidoro-García M, Dávila 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(5):543-8.
4. Oguma T, Palmer L, Birben E, Sonna L, Asano K, Lilly C. Role of prostanoid DP receptor variants in susceptibility to asthma. N Engl J Med. 2004;351(17):1752-63.
5. Oguma T, Asano K, Ishizaka A. Role of prostaglandin D(2) and its receptors in the pathophysiology of asthma. Allergol Int. 2008;57(4):307-12.
6. Kabashima K, Narumiya S. The DP receptor, allergic inflammation and asthma. Prostaglandins Leukot Essent Fatty Acids. 2003;69(2-3):187-94.
7. Arima M, Fukuda T. Prostaglandin D_2 and T(H)2 inflammation in the pathogenesis of bronchial asthma. Korean J Intern Med. 2011;26(1):8-18.
8. Hakonarson H, Bjornsdottir U, Halapi E, Palsson S, Adalsteinsdottir E, Gislason D, et al. A major susceptibility

gene for asthma maps to chromosome 14q24. Am J Hum Genet. 2002;71(3):483-91.
9. Mansur A, Bishop D, Holgate S, Markham A, Morrison J. Linkage/association study of a locus modulating total serum IgE on chromosome 14q13-24 in families with asthma. Thorax. 2004;59:876-82.
10. Sanz C, Isidoro-García M, Dávila I, de Pedro M, Méndez Sde A, Padrón J, et al. A new PTGDR promoter polymorphism in a population of children with asthma. Pediatr Allergy Immunol. 2009;20(2):151-6.
11. Isidoro-García M, Sanz C, García-Solaesa V, Pascual M, Pescador D, Lorente F, et al. PTGDR gene in asthma: a functional, genetic, and epigenetic study. Allergy. 2011;66(12):1553-62.
12. Jamrozik E, Warrington N, McClenaghan J, Hui J, Musk A, James A, et al. Functional haplotypes in the PTGDR gene fail to associate with asthma in two Australian populations. Respirology. 2011;16(2):359-66.
13. Tsai Y, Choudhry S, Kho J, Beckman K, Tsai H, Navarro D, et al. The PTGDR gene is not associated with asthma in 3 ethnically diverse populations. J Allergy Clin Immunol. 2006;118(6):1242-8.
14. Li J, Liu Q, Wang P. Lack of association between three promoter polymorphisms of PTGDR gene and asthma in Chinese Han population. Int J Immunogenet. 2007;34(5):353-7.
15. Li C, Shi L, Yan Y, Gordon B, Gordon W, Wang D. Gene expression signatures: a new approach to understanding the pathophysiology of chronic rhinosinusitis. Curr Allergy Asthma Rep. 2013;13(2):209-17.
16. Lee YH, Choi SJ, Ji JD, Song GG. PTGDR polymorphisms and susceptibility to asthma: a meta-analysis. Mol Biol Rep. 2013;40(3):2195-203.
17. Dreborg S. The skin prick test in the diagnosis of atopic allergy. J Am Acad Dermatol. 1989;21(4 Pt 2):820-1.
18. Muller CR. Quality control in mutation analysis: the European Molecular Genetics Quality Network (EMQN). Eur J Pediatr. 2001;160:464-7.
19. Sreedharan SP, Kumar A, Giridhar P. 3 Biotech (2018) 8: 467. https://doi.org/10.1007/s13205-018-1487-5
20. Rasmussen R. Quantification on the LightCycler. In Meuer S, Wittwer C, Nakagawara K (eds). Rapid Cycle Real-time PCR, Methods and Applications. Springer Press, Heidelberg. 2001:21-34
21. Morey JS, Ryan JC, Van Dolah FM. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biol Proced Online. 2006;8:175-93
22. Livak KJ, SchmittgenTD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta DeltaC(T)) Method. Methods. 2001;25(4):402-8.
23. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-22.
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(2):97-8.
25. Global Iniciative for Asthma. Global Strategy for Asthma Management and Prevention. [Online]. 2014 [Accessed January 26, 2015. Available from: www.ginasthma.org].
26. Valero A, Ferrer M, Sastre J, Navarro A, Monclús L, Martí-Guadaño E, et al. A new criterion by which to discriminate between patients with moderate allergic rhinitis and patients with severe allergic rhinitis based on the Allergic Rhinitis and its Impact on Asthma severity items. J Allergy Clin Immunol. 2007;120(2):359-65.
27. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet. 2013;54(4):391-406.
28. Lopes A, Azenha P, Teodosio C, Inacio M, Silva I, Loureiro G, et al. Impact of allergic rhinitis and specific subcutaneous immunotherapy on peripheral blood. Allergy Asthma Clin Immunol. 2013;9(1):1-10.
29. Li C, Peng J, Ren L, Gan L, Lu X, Liu Q, et al. Roles of histone hypoacetylation in LAT expression on T cells and Th2 polarization in allergic asthma. J Transl Med. 2013;11(26):1-9.
30. Hübner M, Hochhaus G, Derendorf H. Comparative pharmacology, bioavailability, pharmacokinetics, and pharmacodynamics of inhaled glucocorticosteroids. Immunol Allergy Clin North Am. 2005;25(3):469-88.
31. 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.
32. 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.
33. Sonesson A, Bartosik J, Christiansen J, Roscher I, Nilsson F, Schmidtchen A, et al. Sensitization to Skin-associated Microorganisms in Adult Patients with Atopic Dermatitis is of Importance for Disease Severity. Acta Derm Venereol. 2013;93(3):340-5.
34. Singh D, Ravi A, Southworth T. CRTH2 antagonists in asthma: current perspectives. Clin Pharmacol. 2017;9:165-73.

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