

No association of *PTGDR* –441C/T polymorphism with asthma in a North Indian population

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Abstract. *Background:* Asthma is the most prevalent disease in India according to the national survey conducted by NFHS 2 in 1998–399. Prostaglandin D2 (*PGD2*) is a bronchoconstriction inducing metabolite of arachidonic acid in the mast cells, which is produced on exposure to allergens and acts as a ligand for the Prostaglandin D2 Receptor (*PTGDR*). Polymorphisms in the *PTGDR* gene have been suggested to be involved in the mechanism of asthma.

Objective: This is the first study conducted in India, investigating the role of *PTGDR* –441C/T promoter polymorphism in asthma pathogenesis.

Methods: A case-control study was performed with a total of 992 subjects, including 410 adult asthmatics and 582 healthy controls from regions of North India. The *PTGDR* –441C/T polymorphism was genotyped by Tetra-Primer Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra-Primer ARMS PCR).

Results: Statistical analysis of the results between asthma cases and controls for the *PTGDR* –441C/T polymorphism showed $\chi^2 (\chi^2) = 0.29$, OR = 0.95, 95% CI (0.70–1.15) and $p = 0.599$. Neither the genotypic nor the allelic frequencies observed for the *PTGDR* –441C/T polymorphism, were significantly associated with asthma or asthma phenotypes.

Conclusions: The *PTGDR* –441C/T polymorphism is not associated with asthma or its phenotypes in the studied North Indian population.

Keywords: Asthma, Prostaglandin D2 (*PGD2*), Prostaglandin D2 Receptor (*PTGDR*), Tetra-Primer Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra-Primer ARMS PCR)

1. Introduction

Over the last decade, the prevalence of atopic diseases such as asthma, dermatitis and allergic rhinitis has been on a rise globally, and understanding the mechanisms of onset and severity of allergy, has offered a great challenge to researchers and scientists worldwide, due to the complex interplay of genetic as well as environmental factors [6]. According to the second Na-

tional Family Health Survey (NFHS 2), conducted in the year 1998–99, asthma is the most common disease in India as compared to tuberculosis, diabetes, malaria, thyroid and other diseases.

A large number of case-control studies have been conducted in the recent past, all around the world, so as to investigate the role of the various cytokine gene polymorphisms associated with asthma. The results have been found to vary profoundly with differences in the asthmatic populations studied across the world, mostly not revealing similar significances, but leading to a definite conclusion that asthma is a complex polygenic disease, which certainly does not follow classical Mendelian pattern of inheritance [11]. As a result,

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it makes it all the more crucial to identify the genetic makeup associated with the complexity of asthma.

Prostaglandin D2 (*PGD2*) is a major cyclo-oxygenase metabolite of the arachidonic acid in the mast cells which acts as a ligand for the Prostaglandin D2 Receptor (*PTGDR*). The gene for the Prostaglandin D2 Receptor is located on chromosome 14q22.1 in humans and the protein encoded by the gene is a 359 aa, G-protein coupled receptor of 40.276 kDa [12]. It has been observed that on exposure to the allergens, *PGD2* levels increase in asthma patients [13] and is known to induce bronchoconstriction in the lungs [1]. Moreover, a study has revealed that mice devoid of Prostaglandin D2 Receptor (*PTGDR*–/– mice), show minimal infiltration of eosinophils in their lungs, and hence do not develop bronchial hyperresponsiveness (BHR), clearly suggesting that *PGD2* is a mediator in allergic asthma [16].

The –549T/C and –441C/T polymorphisms in the promoter region of the *PTGDR* gene have been suggested to be associated with asthma in a study conducted on American and African American population [18]. In a Spanish population, while the –197T/C promoter polymorphism has been significantly associated with asthma [4], a novel –613C/T polymorphism in the promoter region of the *PTGDR* gene has also been identified, which in diplotype combinations for the –549T/C, –441C/T, –197T/C polymorphisms has been found to be significantly associated with asthma [5]. Moreover, four other novel –338G/A, –731A/G, +6541C/T, +6651C/T *PTGDR* gene polymorphisms were studied in a Danish and UK population. In the UK population, highly significant association was observed between the asthma phenotypic traits and the –731A/G and +6651C/T polymorphisms, while in the Danish population, significance was observed only for the +6541C/T polymorphism [8]. However, no association of any of the *PTGDR* polymorphisms (–549T/C, –441C/T, –197T/C) has been observed with asthma in the studies conducted on asthmatics in the Puerto Rican, Mexican, African American [19], Chinese Han [9] and South Chinese populations [15].

A recent review reported the positive associations of the *PTGDR* gene polymorphisms with asthma among the American white, African American [18] and the Caucasian populations (Spanish, UK and Danish) [4, 5,8] while there was a lack of significant associations from the Asian populations (Chinese Han, South Chinese and Japanese) [7,9,15] as well as the Hispanic population (Puerto Rican and Mexican) [19], indicating the implication of complex genetic and environmental

Table 1
Characteristics of the study population

	Asthma patients 410 (%)	Controls 582 (%)
Sex		
Males	183 (44.6)	351 (60.3)
Females	227 (55.4)	231 (39.7)
Age	38.11 ± 16.20	41.86 ± 16.57
Allergic Rhinitis	323 (78.8)	0
No Rhinitis	87 (21.2)	0
Allergic to atleast		
2 provoking factors	366 (89.3)	0
Non-allergic	44 (10.7)	0
Non- Smoker	345 (84.1)	0
Ever- Smoker	65 (15.9)	0
Spirometry Data (n = 190)		
FVC Observed	2.56 ± 0.96	0
FVC Predicted	3.19 ± 0.73	0
FEV ₁ Observed	1.94 ± 0.82	0
FEV ₁ Predicted	2.68 ± 0.77	0
FEV ₁ /FVC Observed	75.00 ± 13.71	0
FEV ₁ /FVC Predicted	83.12 ± 5.84	0

FVC, Forced Vital Capacity; FEV₁, Forced Expiratory Volume in 1 second.

Spirometry test was conducted for 190 asthma patients.

interactions in the disease, not to forget the marked ethnic differences among the populations [17].

Thus, very contradictory results have been observed for the polymorphisms in the promoter region of the *PTGDR* gene globally, and since no such study had been conducted in the Indian population on this gene, the present pioneer study aimed to investigate the role of *PTGDR* –441C/T polymorphism, inducing a total of 992 subjects, with 410 adult asthma patients and 582 adult healthy controls, from a North Indian population.

2. Methods

Ethical Clearance for conducting the study on human blood samples was granted by the “Ethics Committee, PGIMER, Chandigarh”. The study was conducted strictly in accordance with the ethical guidelines for bio-medical research on human subjects proposed by the “Central Ethics Committee on Human Research (CECHR) ICMR–2000” and of those contained in the “Declaration of Helsinki”. The selection of asthma patients was based on physician’s diagnosis. However, only the patients fulfilling the criteria of GINA (Global Initiative for Asthma) guidelines for diagnosis of bronchial asthma were recruited in the study.

This is the first case-control study conducted in India evaluating the role of *PTGDR* –441C/T polymorphism in asthma pathogenesis by recruiting a total of 992 adult subjects. The patients were recruited from different

states of North India such as Punjab, Haryana, Chandigarh, Uttar Pradesh, Himachal Pradesh, Uttaranchal, Jammu & Kashmir, Rajasthan and New Delhi. A total of 410 asthma patients visiting the Out Patient Department, Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, were enrolled in the study, out of which, 323 subjects were asthma patients with allergic rhinitis. No ABPA (Allergic Bronchopulmonary Aspergillosis) patients were taken in the study. Informed Consent was duly obtained from the asthma patients participating in the study, and a detailed proforma of the asthma patients with a complete questionnaire regarding the clinical symptoms of the disease, i.e. wheeze/whistling, cough, shortness of breath (SOB), allergy, early morning or night symptoms, along with spirometry tests, etc., was assessed. Complete information of the patient regarding name, age, sex, history of the disease, occupation, etc. was taken into account (Table 1). Asthma patients with history of any other pulmonary ailment such as tuberculosis, Chronic Obstructive Pulmonary Disease (COPD), bronchitis, etc., were excluded from the study.

A total of 582 normal and completely healthy controls, with no history of asthma, rhinitis, eczema, allergic skin diseases or any other co-morbid illness were inducted in the study. Some of the healthy volunteers were blood donors at various blood donation camps, educational institutes, employee groups. Care was taken that the control subjects did not have any of the patient conditions in the past. Any subject having a first degree relative with asthma or allergy has not been recruited as a control in the present study. Not only the respiratory or allergic skin disorders, any subject with other diseases such as diabetes, high blood pressure, etc. or with drinking and smoking habits have also not been included as controls in the study. Each control was first enquired for all of the above conditions at the time of taking their written informed consent and before the collection of blood samples.

Blood samples were collected in EDTA coated vials, and stored at -80°C until genomic DNA extraction was done. Genomic DNA was isolated from the thawed blood samples by the Sodium Saline Citrate Buffer Method (FBI Protocol), and checked for DNA on 0.8% agarose gel by electrophoresis.

Genotyping of the PTGDR -441C/T Single Nucleotide Polymorphism (SNP) was carried out by the Tetra-Primer ARMS PCR method as described previously [9]. It is a rapid and sensitive high-throughput assay for the simultaneous detection of both the alleles

in a single PCR using a set of four primers to amplify a larger fragment of DNA with the SNP and the amplicon representing each of the 2 alleles of the gene [14], Forward inner (T allele) 5'-GCCACCCAGTTCAAA CACCAGCACAAT-3', Reverse inner (C allele) 5'-AAGCAGCAGCCACCTG AGAGGAGGGAAG-3', Forward outer 5'-TCTGTAGCTCTGTCACCACGGG CAGATCAC-3' and Reverse outer 5'-CTGACGCGCTGCGTTCTCAGTA GAGACAGA-3'.

PCR was carried out in a thermal cycler, in a total volume of 25 μl containing: 10X PCR Buffer, 3 mM MgCl_2 , 1 mg/ml nuclease free BSA, 50 pmol of each set of primers, 10 mM of each dNTP, 0.125 U Taq polymerase and 2 μl genomic DNA. The PCR conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and final extension step at 72°C for 10 min. The results were observed by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized by UV transillumination. The 434 and 295 bp products in a lane indicated the presence of the wild (C) allele, while 434 and 195 bp products marked the presence of the mutant (T) allele. Heterozygotes were observed as 434, 295 and 195 bp products in a lane (Fig. 1).

The European Molecular Genetics Quality Network (EMQN) good practice guidelines have been followed. A few PCR vials with all the PCR contents except the DNA, were also included per PCR batch as "negative controls". No contamination was observed and there were no "false positives". To minimize the risk of contamination, sterilized and autoclaved solutions and equipments were used during DNA isolation. The ingredients for PCR were well stored at -20°C and were thawed just before use. The patients and controls were not genotyped in separate batches but were randomly analyzed [10]. Retyping of samples was done at random to check for the homology of results.

The genotypic as well as the allelic distribution of the PTGDR -441C/T polymorphism, between the asthma patients and control subjects, were analyzed statistically using Chi^2 (χ^2) test. The data was analyzed with SPSS 17.0 software and Epi Info version 3.4.3. Statistical significance was assumed for p - values < 0.05 .

3. Results

In the present study, a total of 992 subjects, including 410 adult asthma patients and 582 adult healthy con-

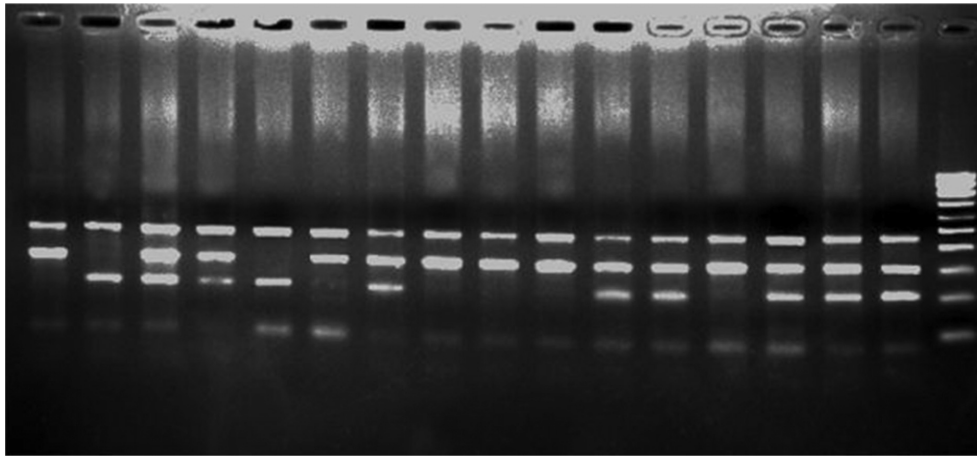


Fig. 1. PCR products of *PTGDR* -441C/T polymorphism on 2% Agarose gel. Lanes 1, 6, 8, 9, 10, 13: Homozygous Wild CC (434 and 295 bp), Lanes 3, 4, 7, 11, 12, 14, 15, 16: Heterozygous CT (434, 295, and 195 bp), Lanes 2, 5: Homozygous Mutant TT (434 and 195 bp), Lane 17: Ladder 100 bp.

trols were genotyped for the *PTGDR* -441C/T polymorphism. Comparing the allelic frequencies in both the groups (Table 2), the wild (C) allele was marginally more prevalent among the asthma patients (66.3%) than in the controls (65.2%), while the mutant (T) allele was slightly more prevalent among the controls (34.8%) as compared to the asthma patients (33.7%), with a χ^2 (χ^2) = 0.29, Odds Ratio = 0.95 with 95% CI (0.70–1.15) and p -value = 0.599.

Further categorizing the asthma patients on the basis of the phenotypic characteristics of the disease (Table 3), as obtained from their detailed proforma, such as sex (male/ female), occurrence (seasonal/ throughout), severity (wheeze on exertion/ wheeze at rest), family history (positive/ nil), rhinitis (positive/ nil), allergy to at least 2 provoking factors (positive/ nil), smoking status (non-smoker/ ever-smoker), longstanding cough (positive/ nil), sputum production (positive/ nil), pattern of daily symptoms (morning SOB/ nocturnal SOB/ anytime during day SOB), no significant association was observed between the *PTGDR* -441C/T polymorphism and asthma phenotypes (all p -values > 0.05).

4. Discussion

PGD2 that acts as a ligand for the *PTGDR* is a major metabolite of arachidonic acid in the mast cells, increases in concentration on exposure to allergens and is directly implicated in the induction of bronchoconstriction in the lungs [1,13]. Moreover, the *PTGDR*^{-/-} mice show minimal infiltration of eosinophils in the lungs, and hence fail to develop any bronchial hyper-

responsiveness (BHR) [16]. The three promoter polymorphisms of the *PTGDR* gene (-549T/C, -441C/T, -197T/C), lead to a difference in the binding and activation by the transcription factors such as c/EBP β , GATA and Sp group members [18], thereby altering the gene expression and leading to an excessive *PTGDR* expression, in turn enhancing asthma susceptibility in response to allergens via *PGD2*.

In the present study, -441C/T polymorphism in the promoter region of the *PTGDR* gene was studied in the adult asthmatic and control population from North India. A total of 992 subjects, with 410 asthma patients and 582 healthy controls were genotyped. As is evident from Table 1, the homozygous wild genotype (CC) was more prevalent among the asthma patients (44.4%) than in the control group (40.4%). The heterozygous genotype (CT) was present more in the controls (49.7%) than in the asthma patients (43.9%), while the homozygous mutant (TT) genotype was marginally more in the asthmatics (11.7%) than in the controls (10.0%). None of the p -values was significant (all p -values > 0.05), indicating no association of the -441C/T SNP with asthma. Moreover, none of the phenotypic traits (Table 2) of the disease showed statistical significance for the studied polymorphism. Hence, neither the genotypic nor the allelic frequencies were associated significantly with asthma or asthma phenotypes.

The results of the present study are in conformity with the results obtained in the study conducted on the Puerto Rican, Mexican and African American population, where no potential association of the -441C/T polymorphism was found with asthma or asthma traits and none of the four studied SNPs

Table 2
Distribution of PTGDR –441C/T genotype and allele frequencies in asthma patients and controls

Genotype frequencies	Asthma patients 410 (%)	Controls 582 (%)	χ^2	OR (95% CI)	p-value
<i>Genotype Frequencies</i>					
CC	182 (44.4)	235 (40.4)		Ref (1.0)	
CT	180 (43.9)	289 (49.7)	2.53	0.80 (0.61–1.06)	0.112
TT	48 (11.7)	58 (10.0)	0.09	1.07 (0.68–1.68)	0.761
CT+TT	228 (55.6)	347 (59.7)	1.59	0.85 (0.65–1.10)	0.207
<i>Allele Frequencies</i>					
C	544 (66.3)	759 (65.2)		Ref (1.0)	
T	276 (33.7)	405 (34.8)	0.29	0.95 (0.70–1.15)	0.599

CC, Homozygous Wild; CT, Heterozygous; TT, Homozygous Mutant; χ^2 , Chi²; OR, Odds Ratio.

Table 3
Phenotypic characteristics of the study population and PTGDR –441C/T polymorphism

Phenotypic traits	n	CC	CT	TT	C	T	χ^2	OR (95% CI)	p-value
<i>Controls</i>	582	235	289	58	759	405			
Males	351	138	180	33	456	246			
Females	231	97	109	25	303	159		Ref (1.00)	
<i>Asthmatics</i>									
<i>Sex</i>									
Males	183	83	82	18	248	118	0.84	0.88 (0.67–1.16)	0.359
Females	227	99	98	30	296	158	0.02	1.02 (0.77–1.35)	0.902
<i>Occurrence</i>									
Seasonal	282	126	127	29	379	185	0.67	0.91 (0.73–1.14)	0.413
Throughout	128	56	53	19	165	91	0.05	1.03 (0.77–1.38)	0.819
<i>Severity</i>									
Wheeze on Exertion	216	95	94	27	284	148	0.04	0.98 (0.77–1.24)	0.842
Wheeze at Rest	194	87	86	21	260	128	0.42	0.92 (0.72–1.19)	0.517
<i>Family History</i>									
Family History (Nil)	285	127	123	35	377	193	0.15	0.96 (0.77–1.19)	0.701
Family History (+ve)	125	55	57	13	167	83	0.23	0.93 (0.69–1.26)	0.631
<i>Rhinitis</i>									
Rhinitis (Nil)	87	37	38	12	112	62	0.05	1.04 (0.73–1.47)	0.829
Rhinitis(+ve)	323	145	142	36	432	214	0.51	0.93 (0.75–1.14)	0.474
<i>Allergy</i>									
Allergy (Nil)	44	19	19	6	57	31	0.01	1.02 (0.63–1.64)	0.934
Allergy to atleast 2 Provoking factors	366	163	161	42	487	245	0.35	0.94 (0.77–1.15)	0.554
<i>Smoking Status</i>									
Non Smoker	345	154	149	42	457	233	0.20	0.96 (0.78–1.17)	0.653
Ever Smoker	65	28	31	6	87	43	0.15	0.93 (0.62–1.38)	0.696
<i>Cough</i>									
Cough (Nil)	74	35	30	9	100	48	0.32	0.90 (0.61–1.31)	0.569
Longstanding Cough (+ve)	336	147	150	39	444	228	0.14	0.96 (0.78–1.18)	0.707
<i>Sputum Production</i>									
Sputum (Nil)	95	43	44	8	130	60	0.75	0.86 (0.61–1.22)	0.386
Sputum (+ve)	315	139	136	40	414	216	0.05	0.98 (0.79–1.21)	0.829
<i>Pattern of Daily Symptoms</i>									
Morning SOB	20	6	13	1	25	15	0.12	1.12 (0.56–2.25)	0.724
Night SOB	292	132	125	35	389	195	0.34	0.94 (0.76–1.17)	0.560
Anytime SOB	98	44	42	12	130	66	0.09	0.95 (0.68–1.33)	0.760

CC, Homozygous Wild; CT, Heterozygous; TT, Homozygous Mutant; χ^2 , Chi²; OR, Odds Ratio; SOB, Shortness of Breath

(-549T/C, -441C/T, -197T/C, +1044G/A), were associated with the disease [19]. Even in a study conducted on a Chinese Han population, none of the three studied polymorphisms (-549T/C, -441C/T, -197T/C), were associated with asthma susceptibility (all *p*- values > 0.05), and it was suggested that none of the three polymorphisms was a risk factor for asthma in the Chinese Han population [9]. In another study conducted in South China, six SNPs (-549T/C, -441C/T, -197T/C, +367C/A, +894G/A and +1044G/A) were genotyped and the findings again did not support *PTGDR* to be a major candidate gene for asthma susceptibility in the Chinese population [15]. Also in a Spanish population, no statistical significance was found either between the allelic distribution of the -549T/C (Fisher's *p*- value = 0.30) and -441C/T (Fisher's *p*- value = 0.41) polymorphisms or between the genotypic distribution of -549T/C (Fisher's *p*- value = 0.51) and -441C/T (Fisher's *p*- value = 0.37) polymorphisms with the asthma phenotype, whereas the -197T/C SNP was significantly associated with asthma [4].

However, the -549T/C and -441C/T polymorphisms were reported to be associated with asthma in the African Americans and American Whites which exposed the carriers to a higher risk of asthma. The study also analyzed the association of haplotype combinations for the -549T/C, -441C/T and -197T/C *PTGDR* polymorphisms and asthma susceptibility in the carriers, where it was observed that the haplotype CCC followed by CCT resulted in an increased expression of the *PTGDR* gene in the carriers, exposing them to a greater risk of asthma whereas the TCT haplotype was associated with low promoter activity and lower risk of asthma [2,3,18]. A similar study was conducted in a Spanish population, wherein the CCC haplotype alone was found to be more common in the asthma patients and the diplotype CCT CCC (-549CC, -441CC and -197TC) was found to be significantly associated with allergic asthma [4]. Another study conducted on Spanish children with asthma analyzed a novel -613C/T polymorphism in the promoter region of the *PTGDR* gene and it was suggested that the heterozygous CT genotype was more common in controls than in the asthma patients. The CCCT CCCC diplotype for the promoter at positions -613, -549, -441, and -197 was more prevalent in asthma patients [5].

Moreover, four other SNPs (-338G/A, -731A/G, +6541C/T, +6651C/T) in the *PTGDR* gene were studied in a Danish and UK population where highly significant associations were observed between the -731A/G, +6651C/T and +6541C/T polymorphisms

and bronchial hyperresponsiveness as well as asthma. The UK population showed highly significant results for the -731A/G and +6651C/T polymorphism while the +6541C/T polymorphism showed significance in the Danish population [8].

In a recent review [17], it has been reported that while association has been observed for the *PTGDR* gene polymorphisms and asthma in the American, African American [18] and Caucasian populations (Spanish, Danish and the UK populations) [4,5,8], no such significance has been observed in the Hispanic (Puerto Rican and Mexican) [19] and Asian (Chinese Han, South Chinese and Japanese) populations [7,9,15].

The reason for the contrasting results of the studies of *PTGDR* polymorphisms association with asthma obtained in different populations globally can be attributed to the differences in the ethnicities as well as to the complex interplay of the multiple genes and environmental factors involved in the mechanism of asthma pathogenesis.

5. Conclusions

The genetic findings of this study expect to throw some light on the much debated role of *PTGDR* gene in asthma and conclude that any alteration in the phenotypic expression of *PTGDR* -441 promoter, has no implication in asthma pathogenesis and *PTGDR* -441C/T polymorphism is not a possible risk factor for asthma in the studied North Indian population.

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