

PTGDR is not a major candidate gene for asthma and atopy in Chinese children

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Airway sensitization requires the expression of prostanoid DP receptor in mice. Recent studies reported that polymorphisms in the gene encoding prostanoid DP receptor (*PTGDR*) were associated with asthma in White people and Black people, but this association could not be replicated among Latinos and Koreans. This study investigated the association between asthma-related traits and six single nucleotide polymorphisms (SNPs) of *PTGDR* in Chinese children, consisted of 308 asthmatics and 368 non-allergic controls. Plasma total and aeroallergen-specific immunoglobulin E were measured by immunoassays. *PTGDR* SNPs were determined by multiplex SNaPshot™ genotyping. All polymorphic markers followed Hardy–Weinberg equilibrium except G1044A in the controls ($p = 0.021$). The linkage disequilibrium (LD) scores for these SNPs were moderate to high, and in particular, T-549C and C-441T were in strong LD. Significant interethnic variations in *PTGDR* alleles and haplotypes (up to 41%) were found in our subjects when compared with White people or Latinos. Asthma diagnosis, atopy and aeroallergen sensitization did not differ among children with different *PTGDR* genotypes ($p > 0.15$ for all). Linear regression showed weakly significant associations between T-197C and G1044A of *PTGDR* and spirometric variables. *PTGDR* haplotypes were not associated with asthma and atopy phenotypes ($p > 0.09$ for all). Our results do not support *PTGDR* to be a major candidate gene for asthma traits in Chinese children.

Ting Fan Leung¹, Chung Yi Li¹, Alice P. S. Kong², Iris H. S. Chan³, Maggie C. Y. Ng², Michael H. M. Chan³, Wing Yee So², Gary W. K. Wong¹, Christopher W. K. Lam³ and Juliana C. N. Chan²

Departments of ¹Paediatrics, ²Medicine and Therapeutics and ³Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong

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Dr Ting Fan Leung, Department of Paediatrics, 6/F, Clinical Sciences Building, Prince of Wales Hospital, Shatin, Hong Kong

Tel.: (852) 2632 2981

Fax: (852) 2636 0020

E-mail: tfleung@cuhk.edu.hk

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Asthma is the most common chronic childhood disease in developed countries (1), and is caused by a complex interplay between a variety of candidate genes and environmental factors. The prostanoid DP receptor gene (*PTGDR*), located on chromosome 14q22.1, encodes a heptahelical transmembrane G-protein-coupled receptor of 359 amino acids (2). *PTGDR* is an asthma candidate gene because it is required for the expression of allergic asthma and airway hyper-reactivity in a mouse model (3). Oguma et al. first reported the relationship between asthma-related traits and single nucleotide polymorphisms (SNPs) and haplotypes of *PTGDR* in White people and African Americans (4). They found

three SNPs in *PTGDR* promoter that had distinct transcription-factor binding motifs. Four functional haplotypes from these SNPs differed significantly in their promoter activities – haplotypes TCT and CCC having the lowest and highest activities respectively. Individual *PTGDR* SNPs and their haplotypes were significantly associated with asthma in both populations.

Subsequent to this study, several groups of researchers tried to replicate the genetic associations in several ethnic groups. Using both the family-based and case–control study design, Tsai et al. (5) studied the same SNP panel in Latinos and African Americans. They could not detect any significant association between *PTGDR*

variants and asthma. SNPs C-441T and T-197C and haplotype TTT showed marginal association with asthma-related traits in Mexican subjects. In another study, Sanz et al. (6) characterized the same three SNPs in the *PTGDR* promoter among 197 Caucasian adults. They found T-197C and the CCT CCC diplotype, formed by the two highest transcriptional efficiency haplotypes, to be significantly associated with asthma. Two other recent studies published conflicting results regarding the association between asthma and *PTGDR* (7, 8). The genetic epidemiology of *PTGDR* SNPs is still not clearly defined. The objective of this study is to assess whether six *PTGDR* SNPs reported in previous studies are associated with asthma and atopy phenotypes in Chinese children.

Patients and methods

Study populations

Unrelated Chinese patients with asthma and aged 5–18 yr old were recruited from pediatric clinics of a university teaching hospital in Hong Kong. Asthma diagnosis was made according to the American Thoracic Society (ATS) guideline (9). Non-allergic controls were recruited from two sources: (i) children attending our Hospital for minor non-allergic and non-respiratory complaints, and (ii) randomly selected schoolchildren who participated in our population-based Hong Kong Secondary School Children Survey (10). All controls gave negative returns for asthma and allergies as evaluated using the standardized ISAAC questionnaire (11), and none of them received any regular drug treatment. Blood samples were collected from the participants during the field study, and stored at -70°C until analysis in a batch for this study. All subjects were free from any infectious symptom for 4 wk before study. The Clinical Research Ethics Committee of our university approved these studies, and subjects and their parents gave informed written consent.

Total and allergen-specific immunoglobulin E in peripheral blood

Total immunoglobulin E (IgE) concentration in peripheral blood was measured by micro-particle immunoassay (IMx analyzer; Abbott Laboratories, Abbott Park, IL, USA) and specific IgE antibodies to *Dermatophagoides pteronyssinus*, cat and cockroaches were measured by fluorescent enzyme immunoassay (AutoCAP; Phadia AB, Uppsala, Sweden). Results on total IgE concentrations were compared with our local

age-specific reference values (upper limits of normal: 120 kIU/l for 5–6 yr; 160 kIU/l for 6–7 yr; 180 kIU/l for ≥ 8 yr) (12, 13). Specific IgE levels for each allergen were categorized into radioallergosorbent test (RAST) classes 0–6 according to manufacturer's instruction. Subjects with RAST class ≥ 3 were considered atopic.

Spirometric assessment

All asthma patients underwent spirometric assessment (COMPACT II; Vitalograph, Buckingham, England) to measure their forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC). Results were compared with local reference values (14).

PTGDR genotyping

The *PTGDR* region of interest in genomic DNA extracted from peripheral blood leukocytes was amplified by a multiplex polymerase chain reaction (PCR) using the following primers: (F) 5'-TCC TCG CCT ATG CAA TGG AG-3' and (R) 5'-GAA CTT CCC GAA GCC CAT GA-3' for 1.1-kb containing T-549C, C-441T, T-197C and C367A (band 1); and (F) 5'-ATG GGT GTT CCA AGC TAA GCT G-3' and (R) 5'-TCC TCA GCT TAC CAC AGA GTG A-3' for 379-bp fragment with G894A and G1044A (band 2). These PCR reactions were performed in a total volume of 10 μl containing 80 ng DNA, 250 μM dNTPs, 1 $\times$ PCR buffer with 1.5 mM Mg, 1.5 pmol of each primer and 0.5 unit of *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany). Samples were denatured at 95°C for 1 min followed by 32 cycles of 95°C for 45 s, 58°C for 60 s, followed by 72°C for 90 s, and a final extension at 72°C for 2 min.

Five microliters of PCR products from each of the above reactions was then incubated with 2 μl Exonuclease I/shrimp alkaline phosphatase (ExoSAP-IT; USB, Cleveland, OH, USA) at 37°C overnight, followed by heating at 80°C for 15 min to inactivate ExoSAP-IT. For *PTGDR* genotyping, 2.5 μl SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA, USA), 1.5 μl pooled and ExoSAP-IT-treated PCR products and 1 μl mixed probes (Table 1) were incubated for 25 cycles at 96°C for 10 s, 54°C for 5 s, and 60°C for 30 s. Finally, 0.6 unit of SAP (USB) was added to the reaction product and incubated at 37°C for an hour to remove the unused primers and unwanted PCR products. SAP was then denatured by heating at 75°C for 15 min. The final reaction mix containing 13.5 μl Hi-Di Formamide, 1.5 μl sample and 1 μl Gene-

Table 1. Experimental details on multiplex genotyping using SNaPshot™, and summary of allele frequencies and Hardy–Weinberg equilibrium (HWE) testing of six *PTGDR* polymorphisms

Probe	Primer sequence	Length (volume)	Minor allele	MAF		p-value for HWE	
				Asthmatics	Controls	Asthmatics	Controls
1 µm probe C (T-549C)	TGA CTG ACT GTG TCT TGA ATT AGC TAG TCA AGG AA	35 mer (10 µl)	C	0.304	0.318	0.986	0.870
1 µm probe B (C-441T)	CTG ACT GAC TGA CCA CCT GAG AGG AGG GCA	30 mer (20 µl)	C	0.321	0.322	0.762	0.753
1 µm probe A (T-197C)	CGG GTC ACA CCC CTC TTT G	19 mer (20 µl)	C	0.018	0.012	0.064	0.972
1 µm probe F (C367A)	TGC ACT CCA GTG CCA TGG CCA GGA	24 mer (10 µl)	A	0	0	NA	NA
1 µm probe E (G894A)	TGA CTG ACT GAC TGA CTG ACT GAC TGG AGG TCT TCT GCT TCT TCA GAG GT	50 mer (10 µl)	A	0	0	NA	NA
1 µm probe D (G1044A)	TGA CTG ACT GAC TGA CGG ATT CCA TGT TAG TGG AAT TGC TGC A	43 mer (10 µl)	A	0.024	0.014	0.146	0.021

MAF, minor allele frequency; NA, not applicable.

Scan-120 LIZ internal size standard was denatured at 95°C 5 min, and the six *PTGDR* genotypes were identified by different fluorescent signals by ABI-310 Genetic Analyzer. Finally, the results of SNaPshot multiplex assay from 40 randomly selected subjects were confirmed by direct sequencing of *PTGDR* PCR products using BigDye Terminator Cycle sequencing kit (Applied Biosystem, Foster City, CA, USA).

Statistical analysis

Results were expressed as proportions or mean and standard deviation. Patient characteristics were compared using the Student's *t*-test or ANOVA for numerical data, and chi-squared or Fisher Exact test for categorical variables. Hardy–Weinberg equilibrium (HWE) for the six *PTGDR* polymorphisms in each group was evaluated by the exact test. To investigate the pattern of linkage disequilibrium (LD) in *PTGDR* locus, pairwise LD coefficient was calculated for each SNP pair by Haploview (Daly Lab, Cambridge, MA, USA), and expressed as *D'* and *r*². The associations between *PTGDR* genotypes and dichotomous phenotypes were analyzed using multivariate logistic regression, and with quantitative traits using linear regression. Logarithmic transformation to base 10 was carried out for total IgE before analysis. Two alternative models (recessive and co-dominant) were fitted for each outcome, adjusting for age and gender as covariates. Finally, *PTGDR* haplotypes were assigned as maximum likelihood estimates using EH program (Rockefeller University, New York, NY, USA). All comparisons were made two-tailed, and *p*-values < 0.05 were considered significant.

Results

Study populations

Three hundred and eight asthmatic patients and 368 controls (178 from hospital clinics and 190 from our community study), were recruited. Table 2 summarizes their demographic and clinical characteristics. The demographic variables of our 190 controls from the community study did not differ significantly from those of the complete cohort (data available upon request). The mean (SD) log-transformed total IgE in our patients and controls were 2.60 (0.61) and 1.80 (0.67), respectively (*p* < 0.0001), but this variable did not differ between controls recruited from our

Table 2. Clinical and laboratory characteristics in our subjects

Characteristics*	Asthmatics (n = 308)	Controls (n = 368)
Age, years	11.4 ± 3.9†	13.5 ± 3.6
Male (n, %)	197 (64.6)†	184 (50.0)
Plasma log-transformed total IgE concentration‡	2.60 ± 0.61†	1.80 ± 0.67
Increased plasma total IgE (n, %),§	235 (77.0)†	101 (27.5)
Atopy (≥one specific IgE) (n, %)	265 (86.9)†	124 (33.8)
IgE sensitization to aeroallergens‡		
<i>Dermatophagoides pteronyssinus</i> (n, %)	263 (86.2)†	120 (32.7)
Cockroaches (n, %)	12 (3.9)†	15 (4.1)
Cat (n, %)	15 (4.9)†	2 (0.5)
Spirometric variables		
FEV ₁ , % predicted	93.8 ± 20.9	ND
FVC, % predicted	111.4 ± 27.0	ND
FEV ₁ /FVC, %	78.9 ± 10.9	ND

ND, not done.

*Expressed in mean ± standard deviation unless stated otherwise.

†*p* < 0.0001.

‡Plasma total IgE levels were measured in 305 patients and 367 controls.

§Compared with the upper limits of local references (120 kIU/l for 5–6 yr; 160 kIU/l for 6–7 yr; 180 kIU/l for ≥8 yr).

Hospital clinics or the community study ($p = 0.785$).

Characterization of *PTGDR* SNPs

The overall genotyping efficiency was virtually 100%, as genotyping failed only on one sample for G1044A. Table 1 summarizes the minor allele frequencies (MAF) and HWE testing for the six *PTGDR* SNPs. All polymorphic markers followed HWE except G1044A in the controls ($p = 0.021$). The LD scores for these SNPs were moderate to high (Fig. 1), and in particular, T-549C and C-441T were in complete LD.

Asthma traits and *PTGDR* polymorphic markers

The relationship between dichotomous asthma-related traits and *PTGDR* polymorphisms was analyzed by logistic regression, and two alternative models (recessive and co-dominant) were fit for each outcome. For all outcomes under asthma diagnosis, atopy and IgE sensitization to individual aeroallergens, the outcome did not differ among subjects with different genotypes of any *PTGDR* SNP under either recessive or co-dominant model ($p > 0.15$ for all). Linear regression models were also fitted with the transformed continuous outcome variables, logarithm of total IgE and spirometric variables

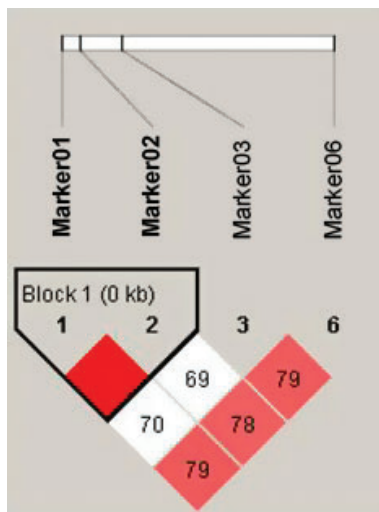


Fig. 1. Linkage disequilibrium (LD) plot among our studied SNPs as analyzed using Haploview. The numbers inside boxes represent D' values for LD. T-549C (marker01) and C-441T (marker02) were in complete LD ($D' = 1.0$ and $r^2 = 0.934$), and T alleles of these SNPs were linked together, and so were their respective C alleles (with frequencies for TT and CC haplotypes being 0.671 and 0.314, respectively). *PTGDR* C367A and G894A were monomorphic and thus not analyzed. Markers '03' and '06' denote T-197C and G1044A respectively.

Table 3. Relationship (p-values) between *PTGDR* genotypes and quantitative traits

SNP*	LR model†	IgE _{log}	FEV ₁ ‡	FEV ₁ /FVC‡
T-549C	Co-dominant	0.477	0.330	0.077
	Recessive	0.879	0.684	0.540
T-197C	Co-dominant	0.769	0.793	0.557
	Recessive	0.058	0.033	0.001
G1044A	Co-dominant	0.769	0.769	0.167
	Recessive	0.096	0.033	0.001

IgE_{log}, log-transformed plasma total IgE concentration; LR, linear regression. *C367A and G894A were not included as these SNPs were monomorphic; and results for C-441T were not shown as the respective p-values were all >0.35 . †p-values were obtained with recessive and co-dominant models, fitting age and gender as covariates.

‡Included data only for asthmatic patients.

(Table 3). The covariates of FEV₁ and FEV₁/FVC were significantly associated with T-197C and G1044A under the recessive model, but not with other *PTGDR* SNP under either co-dominant or recessive model. As only one subject was homozygous for each of the respective minor alleles, we consider that these significant results from regression analyses might be false positives.

Asthma traits and *PTGDR* haplotypes

Only two SNPs (T-549C and C-441T) had MAF $> 5\%$ (Table 1). Three-locus haplotypes were determined from these SNPs and with the addition of T-197C in view of data published recently (7, 8). Table 4 summarizes the relationship between *PTGDR* haplotypes and clinical phenotypes. None of the *PTGDR* haplotypes was significantly associated with asthma-related traits in our Chinese children.

Discussion

Using case-control test of genetic association, this study finds marginally significant association between *PTGDR* SNPs and spirometric parameters in Chinese children. However, these results might be due to small numbers of subjects with the rare genotypes. Haplotype analyses could not detect any significant association between asthma-related traits and *PTGDR* SNPs. Since the initial publication on asthma and *PTGDR* genetics (4), Sanz et al. (6) reported similar disease associations in Spanish and another study on UK and Danish families suggested that three previously unexplored *PTGDR* SNPs at positions -731, +6541 and +6651 were associated with asthma and bronchial hyperresponsiveness (BHR) (7). This latter report, however, did not include the SNPs we genotyped in this study. In contrast, Tsai et al. (5) reported negative results for any

Table 4. Association between asthma and atopy phenotypes and *PTGDR* promoter haplotypes

Phenotype	−549/−441/−197 haplotype	Frequency		OR	95% CI	p-value
Disease status		Asthma	Control			
	TTT	0.674	0.649	1.00		Ref
	CCT	0.290	0.326	0.86	0.56–1.31	0.522
	TCT	0.019	0	NA	NA	0.090
	CCC	0.017	0	NA	NA	0.163
Atopic status		Atopy	No atopy			
	TTT	0.670	0.660	1.00		Ref
	CCT	0.297	0.309	0.94	0.58–1.53	0.875
	TCT	0.017	0	NA	NA	0.342
	CCC	0.013	0.017	0.78	0.13–5.94	0.674
Plasma total IgE		Increased	Normal			
	TTT	0.654	0.687	1.00		Ref
	CCT	0.316	0.278	1.20	0.78–1.86	0.437
	TCT	0.013	0.019	0.70	0.14–3.39	0.722
	CCC	0.012	0.016	0.70	0.11–4.40	0.694
DP-specific IgE		Positive	Negative			
	TTT	0.673	0.653	1.00		Ref
	CCT	0.295	0.317	0.90	0.56–1.46	0.738
	TCT	0.017	0	NA	NA	0.342
	CCC	0.014	0.016	0.81	0.14–6.15	0.682
Cat-specific IgE		Positive	Negative			
	TTT	0.716	0.661	1.00		Ref
	CCT	0.267	0.305	0.78	0.40–1.51	0.526
	TCT	0.017	0.015	1.07	NA	1.000
	CCC	0	0.016	0	0–5.26	0.600
Cockroach-specific IgE		Positive	Negative			
	TTT	0.679	0.664	1.00		Ref
	CCT	0.280	0.306	0.91	0.55–1.51	0.792
	TCT	0.023	0.013	1.92	0.35–9.50	0.406
	CCC	0.018	0.013	1.28	0.17–7.64	0.674

CI, confidence interval; DP, *Dermatophagoides pteronyssinus*; NA, not applicable or not available; OR, odds ratio; Ref, reference haplotype used for comparison.

relationship between asthma traits and *PTGDR* among the Latinos. Kim et al. (8) also could not detect any association between aspirin-intolerant asthma and *PTGDR* SNPs in Korean adults. Our findings add to the literature that *PTGDR* does not appear to be a major candidate gene for asthma-related traits. On the other hand, the frequencies for some of our *PTGDR* alleles were very much different from the published data in Caucasians and Latinos, but similar to those for Koreans.

This study of the genetic epidemiology of *PTGDR* in southern Chinese children shows that the frequencies for alleles −549C, −441C and −197C were 0.318, 0.322 and 0.012. A recent study found identical MAFs (i.e., 0.314, 0.316 and 0.006) for these SNPs in Han (northern) Chinese adults (15). Haplotype frequencies for TTT (0.649 vs. 0.684) and CCC (0 vs. 0.006) were also the same in these Chinese populations. Our results confirm that the frequencies of these SNPs varied widely among different ethnic groups (Fig. 2). We found differences of as high as 41% for minor alleles (for T-549C) and 38% for

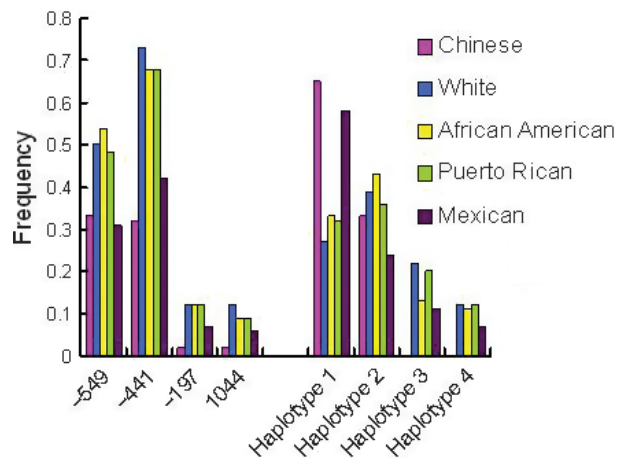


Fig. 2. Comparison of the frequencies for *PTGDR* minor alleles and haplotypes in our Chinese and the published data for other ethnic groups. The minor alleles of SNPs in our subjects were −549C, −441C, −197C and 1044A. Haplotypes 1, 2, 3 and 4 denote TTT, CCT, TCT and CCC, respectively, at positions −549, −441 and −197 of *PTGDR* promoter. The SNPs C367A and G894A were monomorphic (MAF > 99%) and thus not included. Note that data for T-197C, G1044A and the four haplotypes were not available for Koreans (8).

haplotypes (for TTT) between Chinese and White people, and 36% (for C-441T) and 33% (again for TTT), respectively, between Chinese and Latinos (4–6). In particular, the major allele –441C observed in these other ethnic groups became the minor allele in our subjects. This finding is the same as for –52A of *DEFB1* and –159T of *CD14* in Chinese (16, 17). However, our data for T-549C and C-441T were close to those published for Koreans (8). The MAFs for these two SNPs were also similar to those of Mexicans, but much lower than the published values for White people, African Americans or Puerto Ricans. Similarly, the frequency for haplotype TTT of our Chinese was as high as in Mexicans. These results highlight the importance to replicate genetic association studies in each population rather than to generalize the reported results when one try to establish the relationship between susceptibility genes and complex human diseases such as asthma and atopy.

Many genetic association studies failed to show significant results because of inadequate sample size (18). However, we believe that this is unlikely to be the case for this study. We had genotyped 308 ethnic Chinese asthmatics and 368 non-allergic controls. This sample size is comparable to those of published studies (5, 15), and is much larger than another one ($n = 197$) (6). All our subjects were ethnic Chinese, which is again in contrary to the ethnic heterogeneity among subjects under two previous studies (White people vs. African Americans vs. Latinos) (4, 5). The quality of our multiplex genotyping experiments was excellent, with missing data for only one of 4038 genotypes (and the results were also confirmed in a subset by direct *PTGDR* sequencing). Published data from Caucasians suggested that the odds ratios of *PTGDR* SNPs at –549, –441 and –197 for asthma varied between 1.79 and 3.06 (4, 6). Our power calculation reveals that the present sample size has 80% power to detect with 99% confidence an odds ratio for asthma diagnosis of 1.45 for any *PTGDR* SNP at a MAF of 0.3 (i.e., –549C, –441C). This α -level of 0.01 was set to adjust for multiple statistical comparisons. Thus, we believe that this study is sufficiently powered for our objectives, and that *PTGDR* is not associated with asthma traits in Chinese. The recent study on Han Chinese supported our findings (15).

We found marginally significant associations between *PTGDR* and spirometric parameters from linear regression under the recessive model. The result for this latter phenotype had also been observed among Mexicans, although with differ-

ent SNP (5). This finding makes biological sense given the importance of prostaglandin D receptor in recruiting inflammatory leukocytes into the airway and modulating airway muscle tone (19). Our study could not detect any association between plasma total IgE and *PTGDR* SNPs. This result is in keeping with those described by Oguma et al. (4), but is inconsistent with the findings for African Americans (5). The main limitation is that individual studies may be underpowered to detect any association between *PTGDR* and asthma-related traits. As *PTGDR* promoter SNPs at –549, –441 and –197 were included in many studies (4–6, 15), it would be useful to pool the genotype–phenotype data for these SNPs in a meta-analysis to properly answer this research question.

A main drawback of this study relates to the use of different criteria for defining clinical phenotypes. Asthma in our cases was diagnosed by ATS criteria (9), i.e., presence of asthma symptoms together with BHR or reversibility of airflow limitation. On the other hand, our controls were ascertained by negative returns on four items ‘current wheeze’, ‘asthma ever’, ‘rhinoconjunctivitis ever’ and ‘eczema ever’ in the ISAAC questionnaire (11). Following the ISAAC protocol for BHR measurement, de Meer et al. (20) found that 66 of 470 asymptomatic schoolchildren had BHR to hypertonic saline. However, there is no conclusive evidence on how many of these subjects would develop asthma with time. Although objective spirometric parameters were not measured in our controls, the relevant items in ISAAC questionnaire was shown to have >85% sensitivity and 94% negative predictive value for asthma diagnosis (21). We had also validated the clinical utility of Chinese ISAAC questionnaire in our population (22). Despite these findings, we agree that our inclusion criteria would recruit some ‘controls’ with asymptomatic BHR. Nonetheless, there is no relevant data in our population which allows us to estimate statistically the effects of this factor on the current negative genetic association study.

In conclusion, this study reports the genetic epidemiology of six SNPs in *PTGDR* in Chinese. There are significant inter-ethnic variations of its SNPs and haplotypes in our children as compared to White people, Latinos and African Americans. On the other hand, we found only weakly significant associations between this gene and lung function indices. These findings do not support *PTGDR* to be a major candidate gene for asthma-related traits in the Chinese population.

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