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Association of *G-protein-coupled receptor 154* with asthma and total IgE in a population of the Caribbean coast of Colombia

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Summary

Background *G protein-coupled receptor 154* was described as an asthma susceptibility gene by positional cloning. It has been subsequently associated with asthma and other inflammatory diseases in several populations with different ethnic origin. Replication of associations adds reliability to these findings.

Objective To analyze the association of *G protein-coupled receptor 154* with asthma and total and mite-specific IgE levels in a population of the Caribbean Coast of Colombia.

Methods We genotyped seven single nucleotide proteins (SNPs) in *GPR154* in 475 asthmatics, 394 controls and 116 families from Cartagena, Colombia using either SnaPshot or TaqMan.

Total and specific IgE against *Blomia tropicalis* and *Dermatophagoides pteronyssinus* were determined by ELISA. Hardy–Weinberg equilibrium was assessed and case–control and family-based analyses were performed to evaluate the association between the SNPs and their haplotypes and asthma and IgE. Association analyses in the case–control dataset were corrected by population stratification using 52 ancestry informative markers.

Results Allelic distribution was similar to that described in other populations. Two SNPs were associated with the same direction of the effect in both datasets. Allele A of Hpo546333 was protective for asthma (case–control OR: 0.42; 95% CI: 0.17–0.99, $P = 0.042$; $P = 0.043$; families Z score = -2.236 ; $P = 0.025$). Similarly, allele C of rs740347 conferred low risk for asthma (OR: 0.44; 95% CI: 0.28–0.70, $P = 0.00017$; $P_c = 0.00037$) and total IgE (OR: 0.29; 95% CI: 0.09–0.88, $P = 0.015$; $P_c = 0.030$) in the case–control study and families (Z score = -3.207 , $P = 0.0013$; Z score = -3.182 , $P = 0.0014$, respectively). Haplotype CCAGGT was associated with total IgE (OR: 1.76; 95% CI: 1.14–2.71, $P = 0.006$, $P_c = 0.007$) in the case–controls group and CGCGGT with both phenotypes ($P = 0.044$ and $P = 0.032$, respectively) in families. Neither SNPs nor haplotypes were associated with levels of mite-specific IgE.

Conclusions Our findings in a sample of asthmatics from Colombia suggest a relevant role of *G protein-coupled receptor 154* in the pathogenesis of asthma and allergy.

Keywords allergy, asthma, Colombia, *G protein-coupled receptor 154*, immunoglobulin E

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Introduction

Asthma (MIM 600807) is a chronic inflammatory disease affecting millions of people worldwide with a considerable socio-economic impact. It is characterized by reversible airflow obstruction and bronchial hyperresponsiveness, and in a high proportion it is associated with atopy [1, 2]. Linkage and association studies have identified a large number of genes related to asthma pathogenesis; the

interaction of genetic, epigenetic and environmental factors contribute to the onset and the evolution of the disease [3]. Several findings indicate that *G protein-coupled receptor 154* (*GPR154*), could play a role in asthma pathogenesis. This gene, also known as *G protein-coupled receptor for asthma susceptibility* (*GPRA*), *Vasopressin Receptor-Related Receptor 1* (*VRR1*) and *Neuropeptide S Receptor 1* (*NPSR1*), was identified as conferring susceptibility to asthma and high total IgE

levels by genome-wide scan and positional cloning within the locus 7p15–p14 in a Finnish and a Canadian population [4, 5]. Additionally, GPR154 protein isoforms have a distinct distribution in bronchial biopsies from healthy and asthmatic individuals and the murine ortholog of *GPR154* was up-regulated in a mouse model of ovalbumin-induced inflammation [5] suggesting that a gain-of-function mutation in this gene increased the risk of asthma.

One of the fundamentals of genetic research in complex diseases is replicating new findings in populations with diverse ethnic origin and environmental conditions as a support of the original association. In asthma, hundreds of genes have been described but only a few associations have been successfully reproduced in subsequent studies [6–8]. We sought to analyze the association of *GPR154* with asthma, total serum IgE (tIgE) levels and specific IgE levels against two domestic mites by means of a case-control study and a family-based approach in subjects from a city in the north region of Colombia. This tropical country has a prevalence of asthma of 10.4% [2] and 80% of sensitization to mite allergens in asthmatics [9, 10].

Materials and Methods

Subjects

Two independent datasets were used in this study. The first comprised 475 unrelated adult asthmatics (mean age 33.6 ± 18.7 years) and 394 controls (mean age 34.9 ± 17.8 years) and the second included 116 family trios consisting in two parents and one asthmatic proband (348 individuals). The subjects were recruited from the Social Security Clinic and outpatient health centres in Cartagena. This tropical city is located in the province of Bolivar in the Caribbean Coast of Colombia. The asthmatic and control groups were matched by age and gender; the family trios were ascertained through the asthmatic offspring. A full verbal explanation of the investigation was provided and all subjects gave written consent as previously approved by the Bioethics Committee of The University of Cartagena. Asthma was defined according to the Global Initiative for Asthma criteria [11] using a standardized questionnaire previously tested [2, 12] in patients with a history of physician-diagnosed asthma as previously described [13]. Subjects meeting the following criteria were recruited: current asthma, age of 8 years or older having at least 2 years with asthma, three or more episodes of asthma symptoms (wheezing, chest tightness and dyspnoea) in the last 12 months or absence of symptoms because of using anti-asthmatic medications. Controls were individuals with no family or personal history of asthma and allergies. All participants resided in Cartagena, Colombia.

IgE measurements

tIgE levels were measured in all individuals using a commercial enzyme-linked immunosorbent assay (ELISA) kit (RIDASCREEN® Total IgE; R-Biopharm, Darmstadt, Germany). Since *Blomia tropicalis* (Bt) and *Dermatophagoides pteronyssinus* (Dp) are the main source of sensitization in tropical environments [14–16] and, the prevalence of IgE sensitization to other aeroallergens is very low in Cartagena [10], specific IgE levels (sIgE) against these two mite extracts was determined by indirect ELISA in all individuals as described previously [17]. Levels of sIgE above 0.110 optical densities (OD) corresponding to mean OD of five non-allergic controls +3 SD were considered positive. Atopy was defined as positive sIgE to at least one of the two mite extracts tested.

Genotyping

The seven investigated SNPs in *GPR154* (rs323917, rs323922, rs324377, rs324377, rs324377, rs324377 and rs324981) were selected from the 13 SNPs originally reported as 'tagging' most haplotypes in the association study conducted by Laitinen et al. [5]. The aim was to replicate former analyses identifying putative risk alleles and haplotypes [5, 18–22]. These SNPs capture 74% of the information at a $r^2 \geq 0.6$ in the 76.4-kb region of chromosome 7p in HapMap European population. DNA was extracted from peripheral blood samples using standard protocols [23]. Genotyping of the seven SNPs was performed by using either the TaqMan probe-based, 5' nuclease allelic discrimination assay [24] on the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) or PCR and SNaPshot techniques. TaqMan validated assays were designed and manufactured by Applied Biosystems (Applied Biosystems). Automatic calling was carried out with a quality value above 99%. Two SNPs were genotyped using SNaPshot assays with the following primers: for rs323922, forward GGA AGG AAG GTC CCC AAA AT and reverse: GCC AGA AGC TAG TGG TCC TG; for rs324981, forward CTT GCC TGT GAG GGA AAA AG and reverse CAC AGT TGC ACA GGA CCA AG. The amplified fragments had a length of 105 and 345 bp, respectively. To confirm the amplification, PCR products were separated by electrophoresis in 6% polyacrylamide gels [25] and visualized by silver staining [26]. For SNaPshot genotyping, PCR was done using 5 ng of DNA in a 10 µL volume containing 5 µL of 2X QIAGEN Multiplex PCR Master Mix, 1 µL of Primer mix 10× containing 2 µM of each primer. The thermal cycling conditions used were: pre-incubation for 15 min at 95 °C; followed by 30 cycles of 30 s at 94 °C, 90 s at 60 °C, 60 s at 72 °C; and a final incubation step of 72 °C for 10 min. PCR amplification protocols were optimized in a PCR System 2400 Thermal Cycler (Applied Biosystems). Detection of alleles was done

with SNaPshot multiplex technique. The PCR amplification mix product was purified using ExoSAP-IT (USB corporation, Cleveland, Oh, USA) at 37 °C for 15 min and 85 °C for 15 min and then 1.5 µL of this PCR product was added to 1 µL SNaPshot mix, 1 µL of H₂O and 1.5 µL SBE primers (0.2 µM). Thermal cycling conditions were 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 30 s at 60 °C. The SNaPshot reaction products were purified with 1 U of shrimp alkaline phosphatase at 37 °C for 1 h and 85 °C for 15 min and electrophoresed in ABI PRISM 3101™ sequencer (Applied Biosystems). Gene Scan software version 2.1 was used to assign genotypes. Approximately 10% of samples were repeated for quality control with an average error rate of 0.1%. Mendelian inconsistencies were identified using Sib-Pair 1.00a17 (available at <http://www.qimr.edu.au/davidD/#sib-pair>) with 0.3% discordant genotype pairs in the family datasets. Genotypes were made missing as appropriate for members of nuclear families involved, which rendered seven genotypes (0.2%) missing for further analyses. Approximately 8% of the samples were genotyped using both TaqMan probe-based, 5' nuclease allelic discrimination assay and PCR and SnaPshot technique with a 100% of reproducibility of the results.

Statistical Methods

The analyzed phenotypes were asthma, sIgE and tIgE. Asthma status was considered as a dichotomous trait (i.e., presence/absence) and sIgE against Bt and Dp as continuous variables. tIgE was log transformed (log tIgE), percentiles were calculated based on all available subjects with existing tIgE measurements ($N = 1217$) and log tIgE 90th percentile (3.12 UI/mL) was used as dependent variable (above/below 90th percentile) with two categories denoted as 'high' and 'low' tIgE levels, respectively. Comparisons of the demographic characteristics among cases and controls and among cases and family probands were done by means of Student's *t*-test and χ^2 test as needed. Departure from Hardy-Weinberg equilibrium proportions of each SNP was tested among controls and parents. Fisher's exact test was used to compare allelic frequencies between the cases vs. controls and between individuals with high and low tIgE levels. Logistic regression was performed to model the effect of each genotype on asthma status and tIgE levels (high or low) compared with a reference genotype, including age and sex as covariates; linear regression was used to analyse sIgE levels as outcome variable under the same model. For SNP 740347, a dominant model was also analysed in this database. Linkage disequilibrium among the SNPs was calculated in the total sample ($N = 1217$) using the normalized measure of allelic association D' [27] implemented in Haploview (<http://www.broad.mit.edu/mpg/haploview>). Since we sought to replicate previous analysis of associa-

tion of *GPR154* haplotypes and asthma [19] we estimated haplotypic frequencies in asthmatics, controls and in individuals with high and low tIgE levels using the expectation-maximization algorithm of Slatkin and Excoffier [28] using ARLEQUIN software [29]. In the haplotypic analysis, each individual contributed two haplotypes (one from each chromosome) to the analyses and the frequency and estimated counts of each haplotype were then assessed against all others using standard procedures for odds ratio (OR) and confidence interval (CI) calculations. All calculations were done with Statistical Package for the Social Sciences software (SPSS 16 for Windows, SPSS Inc, Chicago, IL, USA). In the family trios, tests of linkage and association between each SNP and their haplotypes, and asthma, high tIgE and sIgE against Bt and Dp were conducted using the family-based association test (FBAT Version 2.0.2, available at: <http://www.biostat.harvard.edu/~fbat/default.html>) [30]. Association tests between the six polymorphisms and the phenotypes were performed under an additive model for alleles and dominant and general genotype model for genotypes. For haplotype analyses, haplotype frequencies were obtained by an expectation-maximization algorithm, which maximizes the likelihood of phased haplotype frequencies based on all observed genotypes in the nuclear family and haplotype permutation tests were performed to determine association of haplotypes with the analyzed traits.

Correction by population stratification

A panel of 52 ancestry informative markers (AIMs) was also genotyped in the total sample. These markers showed large differences in frequency between the parental populations and were used to control for the presence of genetic structure due to admixture [31]. To test for association of the SNPs in the candidate genes within the phenotypes correcting by genetic population structure, we used the program ADMIXMAP (available at <http://www.lshtm.ac.uk/eu/genetics/index.html#admix>). This is a general purpose program for modeling population admixture with genotype and phenotype data, based on a combination of Bayesian and classical methods (see Supporting Information). The statistical algorithms used in this program have been described in detail previously [32, 33] and its efficacy to adjust association analysis of candidate genes by individual admixture has been demonstrated for another traits [34]. For this analysis, the Cartagena population was modeled as formed by admixture between three subpopulations: European, Native American and West African. Score tests for allelic association with the traits, conditional on individual admixture and age and sex as covariates were constructed by testing the coefficient *b* for the effect of the allele under study (coded as 0, 1, or 2 copies) in a regression model. An

approximation to the maximum likelihood estimate of the effect size was obtained from the score test by dividing the score by the observed information. A P -value below 0.05 (P_c) was considered statistically significant. Bonferroni's correction for multiple tests was performed; after six independent tests, a single test would be significant at the 0.05 nominal level with a P -value <0.008 .

Results

Demographic and clinical characteristics of case-control dataset (475 asthmatics/394 controls) and the 116 family trios are presented in Table 1. Log tIgE and sIgE levels were significantly higher in cases than controls ($P < 0.01$). Most asthmatics were atopic in both the case group (83.3%) and the offspring group (78.4%). Asthmatic offspring were younger than the cases ($P < 0.001$); total and specific IgE levels were similar among the two groups (mean tIgE $\pm$ SD: 2.7 ± 0.46 vs. 2.7 ± 0.50 , $P = 0.725$; mean sIgE Bt $\pm$ SD: 0.88 ± 1.09 vs. 0.95 ± 1.13 , $P = 0.539$; mean sIgE Dp $\pm$ SD: 0.56 ± 0.81 vs. 0.53 ± 0.80 , $P = 0.678$), Table 1.

Figure 1a shows the position of the seven SNPs selected for genotyping located in intron two and exon three (76.4 kb) of the *GPR154* gene on chromosome 7p. They were chosen because they tagged most common haplotypes in the 77 kb chromosomal region originally identified as a susceptibility locus for asthma and high total IgE in a Finnish and a French-Canadian population [4]. Rs324957 was excluded from the analysis because of significant deviation from Hardy-Weinberg equilibrium (data not shown) and displayed variable pairwise D' values ranging from 0.02 to 0.92 in the total sample. As shown in Fig. 1b, three SNPs were in a single 14 kb haplotype block using the algorithm based on solid spine of LD; no blocks were found using the algorithm of Gabriel et al. [35].

The allelic distribution of the six SNPs in the controls from Cartagena was similar to that described in Finland/Canadian, German, Chinese, Mexican, Italian and Eur-

opean populations (Table 2). Table 3 displays allelic and genotypic frequencies in asthmatics and controls and results of single SNPs association tests with asthma and high tIgE levels (90th percentile) in the case-control dataset. Allele A of hopo546333 conferred protection for asthma (OR: 0.42; CI 95%: 0.177–0.99, $P = 0.042$); this association was also significant after correction by population stratification ($P_c = 0.043$) but not after Bonferroni's correction. The variant C of rs740347 was negatively associated with both asthma (OR: 0.44, CI 95%: 0.28–0.70, $P = 0.17 \times 10^{-3}$) and high tIgE levels (OR: 0.29, CI 95%: 0.09–0.88, $P = 0.015$); the significant P -values held up after correcting by population stratification ($P_c = 0.00037$ and $P_c = 0.030$, respectively). CC+CG genotypes of rs740347 were also significantly associated with asthma ($P = 0.8 \times 10^{-5}$) and high tIgE levels ($P = 0.023$). Only the association of this SNP with asthma remained significant after applying adjustment by multiple comparisons. No associations were found between any single SNP and levels of sIgE against Bt or Dp in the case-control analysis neither before nor after correction by population structure (data not shown).

Results of the family-based association tests of the polymorphisms with asthma and high tIgE levels under the additive (allele) and general genotype models are presented in Table 4. Although the complete dataset ($N = 348$ individuals) was included in each analysis, the number of informative families varied for each SNP depending on the allelic frequencies. Variant A of hopo546333 was significantly associated with a lower risk for asthma (Z score: -2.236 , $P = 0.025$) and high tIgE levels (Z score: -2.121 , $P = 0.034$); since minor allele frequency of this SNPs was very low (0.02), a small number of families were informative in the analysis (Table 4). Allele C of rs740347 was also negatively associated with asthma (Z score -3.207 , $P = 0.0013$) and high tIgE levels (Z score -3.182 , $P = 0.0014$). Under a dominant model, rs740347 CC+CG genotypes were associated with asthma (Z score -3.221 , $P = 0.0013$) and high tIgE levels ($P = 0.0010$). In this dataset, allele C of rs324377 was associated with a high risk for asthma (Z score: 2.357,

Table 1. Demographic and laboratory characteristics of the subjects in the case-control and family based datasets from Cartagena, Colombia

Characteristics	Case control study			Family study	
	Cases $N = 475$	Controls $N = 394$	P value	Probands $N = 116$	Parents $N = 232$
Age (means $\pm$ SD, years)	33.6 $\pm$ 18.7	34.9 $\pm$ 17.8	0.275	17.2 $\pm$ 9.9	46.5 $\pm$ 11.1
Male gender, n (%)	189 (39.8)	170 (43.1)	0.317	58 (50.0)	116 (50.0)
Atopy, n (%)	396 (83.3)	12 (3.04)	<0.001	91 (78.4)	114 (49.1)
Log total IgE (means $\pm$ SD, IU/mL)	2.7 $\pm$ 0.46	1.9 $\pm$ 0.54	<0.001	2.7 $\pm$ 0.50	2.32 $\pm$ 0.53
Total IgE above 90 th percentile, n (%)	107 (22.5)	2 (0.5)	<0.001	24 (20.6)	4 (1.7)
sIgE against Bt (means $\pm$ SD, OD)	0.88 $\pm$ 1.09	0.094 $\pm$ 0.42	<0.001	0.95 $\pm$ 1.13	0.26 $\pm$ 0.50
sIgE against Dp (means $\pm$ SD, OD)	0.56 $\pm$ 0.81	0.096 $\pm$ 0.61	<0.001	0.53 $\pm$ 0.80	0.20 $\pm$ 0.33

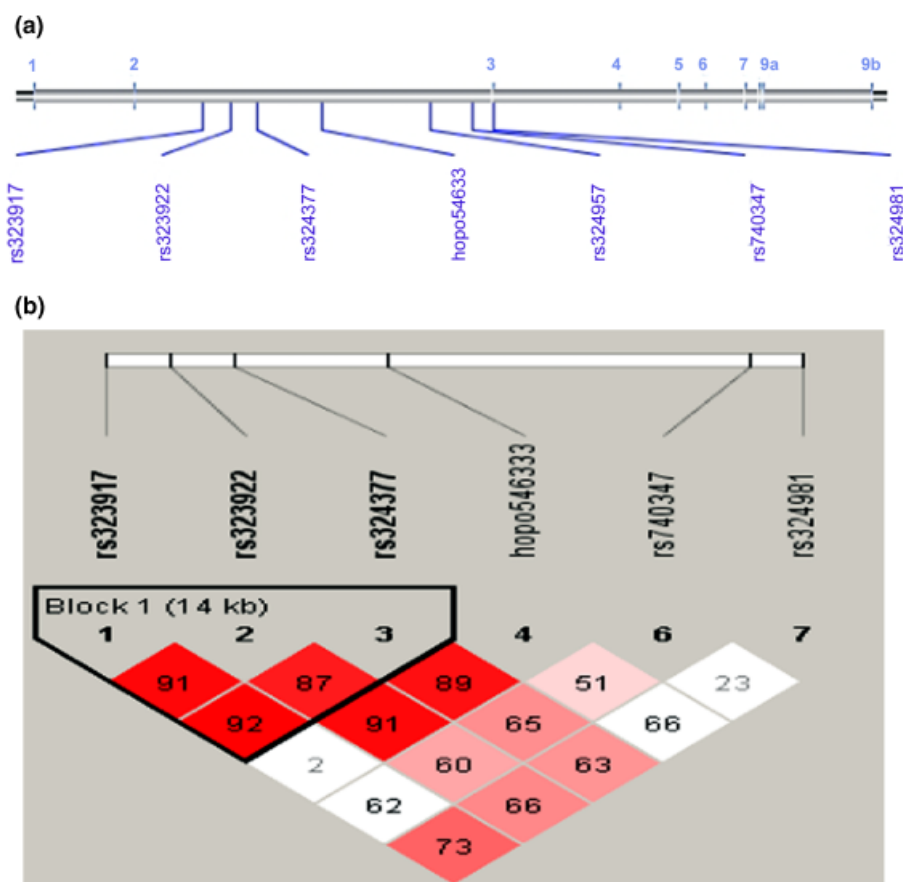


Fig. 1. Schematic genetic structure of *GPR154* gene and position of the genotyped SNPs. (a) Overview of the exon/intron distribution of the *GPR154* gene located on chromosome 7p and location of the seven SNPs selected for genotyping. Exons are shown as blue vertical bars and introns as gray horizontal bars. The chromosomal position of these SNPs (reference contig NT 007819.16) is as follows: 34708168 (rs323917, C/G), 34715307 (rs323922, G/C), 34722500 (rs324377, C/A), 34739277 (hopo54633, G/A), 34778827 (rs740347, G/C) and 34784638 (rs324981, A/T). A, adenine; C, Cytosine; G, guanine; T, thymine; and rs, database SNP accession number. (b) The 76.4-kb region containing haplotype blocks in the *GPR154* gene as defined by solid spine of LD. rs324957 was excluded from this analysis because of significant deviation from Hardy-Weinberg equilibrium in controls and parents. The numbers in each box correspond to the pairwise linkage disequilibrium coefficient D' between respective SNPs. All the subjects included in the case-control groups and families were analyzed together ($N = 1217$). The SNPs are ordered according to their position in the gene (see a).

$P = 0.018$) and high tIgE levels (Z score: 2.201, $P = 0.027$). After correcting by multiple comparisons, allele C and the grouped genotypes CC+CG of rs740347 remained associated with asthma and high tIgE in the family-based analysis. No associations were found between any single SNP and levels of sIgE against Bt or Dp in the case-control analysis neither before nor after correction by population structure (data not shown).

Estimated frequencies of haplotypes in the total analyzed sample are shown in Table 5. Technical reasons precluded the genotyping of two SNPs (rs324384 and rs324396) which were included in the haplotypes described by Laitinen et al. [5]. For that reason, the haplotypes displayed in Table 5 do not match up with those described in the original study and their frequencies are not susceptible to compare with each other. However, since the other four SNPs are common between the studies, H4/H5 and H1/H2 haplotypes

found in our study can correspond to Laitinen's H3 and H6, respectively. The results of the haplotype association analyses with asthma and high tIgE levels in the case-control group and the families are shown in Table 5. Positive associations between H2 haplotype (CGCGGT) and asthma and high tIgE levels were observed in the family-based analysis ($P = 0.044$ and 0.032, respectively; Table 5). Although in the case-control group no associations between haplotypes and asthma were found, H4 (CCAGGT) was significantly associated with high tIgE levels in this dataset (OR: 1.76, CI 95%: 1.14–2.71, $P = 0.006$, $P_c = 0.007$).

Discussion

In this study, we analyzed the association of six polymorphisms and their haplotypes in *GPR154* gene with

Table 2. Comparisons of the allelic distribution of *GPR154* polymorphisms in controls from Cartagena and other populations

SNP/Allele		Cartagena Cases/Controls (<i>N</i> = 475/ <i>N</i> = 394)	North European (<i>N</i> = 221) [21]	Mexicans (589 nuclear families) [39]	Finland/French Canadians (<i>N</i> = 499/ <i>N</i> = 402) [5]	Italians (<i>N</i> = 927) [40]	Germans (<i>N</i> = 3.063) [19]	Finland neonates (<i>N</i> = 87) [18]	Chinese (<i>N</i> = 232) [22]	Europeans PARSIFALN = 3113/BAMSE <i>N</i> = 686) [19]
rs323917 (C/G)	G	0.08/0.06	0.06	NA	0.05/0.06	0.07	0.06	0.08	NA*	0.06/0.07
rs323922 (C/G)	C	0.42/0.39	0.39	NA	0.46/0.43	0.42	0.42	0.38	0.48	0.42/0.45
rs324377 (A/C)	A	0.43/0.41	0.44	NA	0.47/0.44	0.43	0.45	0.56	NA	0.44/0.48
Hopo546333 (G/A)	A	0.008/0.02	0.07	NA	0.12/0.06	0.06	0.09	NA	NA	0.09/0.08
rs324957 (A/G)	G	0.40/0.42	NA	NA	0.47/0.47	NA	NA	NA	0.53	NA
rs740347 (C/G)	C	0.03/0.08	0.11	0.05	0.22/0.13	0.13	0.16	0.08	0.09	0.15/0.14
rs324981 (A/T)	A	0.42/0.41	NA	0.30	0.47/0.47	NA	NA	NA	0.49	NA

*NA, not available.

Table 3. Association between the *GPR154* polymorphisms and asthma and high tIgE levels (90th percentile) in the case-control dataset from Cartagena, Colombia

SNP	Allele/ genotype	Cases (%)*	Controls (%)*	Asthma OR (CI, <i>P</i> -value)	High tIgE levels OR (CI, <i>P</i> -value)
rs323917	C	871 (0.92)	721 (0.94)	1.30 (0.88–1.92, 0.164)	1.31 (0.74–2.30, 0.322)
	G	77 (0.08)	49 (0.06)		
	CC [†]	398 (0.84)	338 (0.88)	1.0	1.0
	CG	75 (0.15)	45 (0.11)	1.42 (0.93–2.15, 0.08)	1.22 (0.65–2.27, 0.50)
	GG	1 (0.002)	2 (0.005)	0.42 (0.02–5.97, 0.47)	3.13 (0.62–15.70, 0.20)
rs323922	G	551 (0.58)	478 (0.60)	1.12 (0.92–1.3, 0.261)	1.01 (0.73–1.39, 0.94)
	C	399 (0.42)	310 (0.39)		
	GG [†]	85 (0.18)	53 (0.13)	1.0	1.0
	CG	229 (0.48)	204 (0.52)	0.70 (0.46–1.05, 0.07)	0.91 (0.55–1.51, 0.71)
	CC	161 (0.33)	137 (0.35)	0.73 (0.48–1.13, 0.13)	1.07 (0.54–2.11, 0.82)
rs324377	C	536 (0.57)	419 (0.59)	1.05 (0.86–1.29, 0.61)	0.95 (0.68–1.32, 0.75)
	A	392 (0.43)	291 (0.41)		
	CC [†]	161 (0.35)	123 (0.35)	1.0	1.0
	AC	214 (0.46)	173 (0.49)	0.95 (0.69–1.30, 0.72)	0.96 (0.57–1.60, 0.85)
	AA	89 (0.19)	59 (0.16)	1.15 (0.75–1.76, 0.49)	0.90 (0.45–1.81, 0.76)
hopo546333	G	930 (0.99)	731 (0.98)	0.42 (0.177–0.99, 0.042)	0.0 (0.0–1.72, 0.087)
	A	8 (0.008)	15 (0.02)		
	GG [†]	461 (0.98)	359 (0.96)	1.0	1.0
	AG	8 (0.02)	13 (0.04)	0.48 (0.18–1.25, 0.09)	0.0 (0.0–1.87, 0.10)
	AA	0	1	0.0 (0.0–13.53, 0.25)	0.0 (0.0–135.4, 0.71)
rs740347	G	909 (0.97)	702 (0.92)	0.44 (0.28–0.70, 0.00017)	0.29 (0.09–0.88, 0.015)
	C	33 (0.03)	58 (0.08)		
	GG [†]	442 (0.93)	326 (0.85)	1.0	1.0
	CG	25 (0.05)	50 (0.13)	0.37 (0.22–0.62, 0.00006)	0.31 (0.08–1.05, 0.040)
	CC	4 (0.008)	4 (0.010)	0.74 (0.15–3.52, 0.66)	0.0 (0.0–5.06, 0.29)
rs324981	T	550 (0.58)	461 (0.58)	1.02 (0.84–1.24, 0.83)	1.05 (0.76–1.44, 0.77)
	A	398 (0.42)	327 (0.42)		
	AA [†]	168 (0.35)	129 (0.32)	1.0	1.0
	AT	214 (0.45)	203 (0.52)	0.81 (0.59–1.10, 0.16)	0.70 (0.42–1.15, 0.13)
	TT	92 (0.19)	62 (0.15)	1.14 (0.75–1.73, 0.52)	0.95 (0.50–1.80, 0.86)

*Because of genotyping failure, the case and control numbers for genotypes in each SNP may be < 475 and 394, respectively.

[†]Each genotype in every SNP is compared to a reference genotype.

asthma and total and specific IgE levels in a sample from Cartagena, Colombia using two different approaches, a case-control and a family-based association analysis. This gene has been previously associated with asthma, IgE

levels, wheezing, airway hyperresponsiveness, allergic sensitization, inflammatory bowel disease, respiratory distress syndrome and mental disorders in independent studies in ethnically different populations [4, 18, 19, 22,

36–38], Table 6. However, in a population of northern European descent and a Mexican population, no significant association was found with atopic dermatitis [21] and

Table 4. Family based association tests for the *GPR154* polymorphisms and asthma and high tIgE levels (90th percentile) in a population of Cartagena, Colombia

SNP	Allele/ genotype	No. of informative families	Asthma		High tIgE levels	
			Z score	FBAT P value	Z score	FBAT P-value
rs323917	C	30	1.095	0.27	0.745	0.456
	G	30	−1.095	0.27	−0.745	0.456
	CC	30	1.095	0.27	0.745	0.456
	CG	30	−1.095	0.27	−0.745	0.456
	GG	0	–	–	–	–
rs323922	C	72	−1.897	0.057	−1.698	0.089
	G	72	1.897	0.057	1.698	0.089
	CC	34	−1.289	0.19	−1.308	0.190
	CG	72	−0.471	0.63	−0.183	0.855
	GG	56	1.533	0.12	1.257	0.208
rs324377	A	60	−2.357	0.018	−2.201	0.027
	C	60	2.357	0.018	2.201	0.027
	AA	31	−1.705	0.088	−1.828	0.067
	AC	60	−0.258	0.796	0.000	1.000
	CC	41	1.784	0.074	1.476	0.140
hopo546333	A	5	−2.236	0.025	−2.121	0.034
	G	5	2.236	0.025	2.121	0.034
	AA	0	–	–	–	–
	AG	5	−2.236	0.025	−2.121	0.033
	GG	5	2.236	0.025	2.121	0.033
rs740347	C	13	−3.207	0.0013	−3.182	0.0014
	G	13	3.207	0.0013	3.182	0.0014
	CC	1	−0.577	0.563	−0.577	0.563
	CG	13	−30.57	0.002	−3.024	0.002
	GG	13	3.221	0.001	3.272	0.001
rs324981	A	68	0.436	0.66	−0.253	0.800
	T	68	−0.436	0.66	0.253	0.800
	AA	45	0.781	0.434	0.413	0.679
	AT	68	−0.728	0.466	−0.928	0.353
	TT	39	0.169	0.865	0.863	0.388

childhood asthma, respectively [39]. Our results strongly support the important role of this gene in asthma and other inflammatory allergic diseases.

In our sample, we found a negative significant association between allele C rs740347 and asthma and high tIgE levels in both datasets. The association with asthma remained significant after controlling by the effect of population stratification and applying Bonferroni correction. Additionally, allele A of hopo546333 was associated with low risk for asthma in the case–control study even after adjustment by differences in admixture proportions. Consistently, the protective effect for both asthma and tIgE levels was also observed in the Cartagena families. Allele C of rs324377 was significantly associated with a higher susceptibility for asthma and high tIgE in the family-based analysis. These two latter associations did not hold after correcting by multiple testing. Rs740347 and hopo546333 were previously related with asthma and related phenotypes in a German [20] and an Italian population [40]; although the associated alleles in Germans had opposite effects compared with those found in our study (Table 6). In a cohort of European asthmatic children, a strong interaction was observed between those SNPs and the timing of initiation of current regular contact to farm animals, which was a surrogate phenotype representing exposure to lipopolysaccharides [41]. Our case–control study had a power of 82% to detect a minimum OR of 1.8 with a MAF of 0.05 [42], however, we consider that it was still underpowered to detect the association of hopo546333 with high tIgE levels given the low minor allele frequency in our population. Even though our findings were obtained with a small sample size for some SNPs given their allelic frequency, they are consistent with previous associations observed in several populations (Table 6). A replicated genetic effect means that the genetic variants can have a common, consistent, final biological effect on individuals [43]. One strength of our study is that we consistently observed the effect of

Table 5. Association between haplotypes in *GPR154* and asthma and high total IgE levels (90th percentile) in the case–control and the family based datasets from Cartagena, Colombia

Frequency Haplotypes*			Case/control study (N = 475/N = 364) OR (95%CI, P value)		Family based association study (116 family trios/N = 348) Z Score, P-value (Permutation test P-value)		
			Asthma	High total IgE levels	No. of informative families	Asthma	High total IgE levels
H1	CGCGGA	0.31	0.97 (0.77–1.21, 0.75)	1.06 (0.76–1.49, 0.71)	42	1.954, 0.050 (0.027)	1.788, 0.073 (0.049)
H2	CGCGGT	0.20	1.02 (0.78–1.33, 0.88)	0.79 (0.51–1.19, 0.23)	21	2.010, 0.044 (0.048)	2.137, 0.032 (0.045)
H3	CCAGGA	0.18	1.04 (0.80–1.35, 0.78)	0.96 (0.64–1.44, 0.83)	7	−1.214, 0.22 (0.21)	−1.046, 0.29 (0.17)
H4	CCAGGT	0.11	0.86 (0.66–1.10, 0.21)	1.76 (1.14–2.71, 0.006)	26	−1.195, 0.23 (0.22)	−0.422, 0.672 (0.83)
H5	GGCGGT	0.03	1.29 (0.74–2.25, 0.34)	0.92 (0.45–1.88, 0.82)	NA	NA	NA
H6	GCAGGT	0.03	1.13 (0.55–2.36, 0.72)	1.18 (0.52–2.68, 0.70)	18	−0.229, 0.81 (0.99)	0.180, 0.85 (0.72)

*All the subjects included in the case–control group and families were analysed together (N = 1217).

Table 6. Published replication studies examining associations of *GPRA154* SNPs and haplotypes with asthma or asthma related phenotypes

Population	Phenotype	SNP or haplotype	Allele/ genotype	RR/OR (95%CI); Z score	P value
Finland (106 Families; 31 trios)/ Canadian (193 trios) [5]	Asthma or high IgE	rs323922	C	–	0.004
	Asthma	H2	–	2.5 (2.0–3.1)	0.0009
	High IgE	H4,H5	–	1.4 (1.1–1.9)	0.01
Italians (117 families, <i>N</i> = 927) [40]	High IgE	hopo546333	NA	NA	0.0046
		rs740347	NA	NA	0.006
		H4	–	–3.024	0.0025
Italians (59 nuclear families) [40]	Asthma	H5	–	–2.23	0.025
		H6	–	2.657	0.007
		hopo546333	A	1.40 (1.04–1.88)	0.025
Germans (<i>N</i> = 3.063) [20]	Asthma	rs324396	T	0.77 (0.61–0.7)	0.027
		rs740347	C	1.34 (1.04–1.72)	0.022
		H4	–	1.83 (1.08–3.08)	0.024
	Asthma + BHR	hopo546333	A	2.38 (1.22–4.66)	0.009
		rs740347	C	2.71 (1.45–5.09)	0.001
		H4	–	3.51 (1.08–11.4)	0.036
	High IgE	rs324396	TT	0.33 (0.12–0.93)	0.028
		rs740347	C	1.63 (1.10–2.42)	0.015
	BHR	rs324981	AA	0.58 (0.37–0.91)	0.02
		rs324987	TT	0.56 (0.36–0.88)	0.01
	BHR	rs324981	AA	0.61 (0.44–0.85)	0.004
	Allergic sensitization	rs324384	C	0.88 (0.80–0.98)	NA
Europeans PARSIFAL (<i>N</i> = 3113)/ BAMSE (<i>N</i> = 686) [19]		rs324396	T	0.88 (0.79–0.99)	NA
	Allergic asthma	rs324384	C	0.81 (0.66–1.00)	NA
		rs324396	T	0.78 (0.62–0.98)	NA
		H1		0.78 (0.63–0.96)	0.045
		H5		1.47 (1.01–2.14)	0.023
	Asthma	rs324396	T	0.83 (0.70–0.97)	0.02
	Allergic rhinoconjunctivitis	rs324384	C	0.83 (0.66–1.04)	NA
		H3	–	0.68 (0.48–0.96)	0.046
	Asthma; SPT	No SNPs or haplotypes associated			
Mexicans (589 nuclear families), [39]	Asthma	rs323922	NA	NA	NS
	Asthma	rs2609234	NA	NA	0.03
		rs1006392	NA	NA	0.03
		rs714588	NA	NA	0.03
		rs1379928	NA	NA	0.003
		rs963218	NA	NA	0.04
	BHR	rs323917	NA	NA	0.02
		rs10278663	NA	NA	0.007
	Total IgE	rs1860370	NA	NA	0.04
	Asthma	rs2609234	NA	NA	0.01
		rs714588	NA	NA	0.01
		rs1379928	NA	NA	0.0006
CAMP (Families of 497 children) [37]		rs323922	G	0.93 (0.88–1.0)	0.040
	Wheezing	rs323922	G	0.93 (0.87–1.0)	0.048

SPT, skin prick test to allergens; BHR, bronchial hyperresponsiveness; NA, not available; CAMP, Childhood Asthma Management Program; RR, relative risk; OR, odds Ratio; CI, confidence interval.

rs740347 and hopo546333 in our two datasets comprised of adult cases and teenager offsprings which makes our result generalizable to childhood asthma as well as adult asthma. The intronic position of most of the SNPs point them out as potential targets for functional analysis exploring their role as transcription factor binding, reg-

ulation of alternative splicing sites in the gene. Analyses *in silico* indicate that the majority of them have an unknown predicted function and for rs324377 and rs324957 there is a low–low probability of functioning as enhancer binding sites (http://fastsnp.ibms.sinica.edu.tw/pages/input_CandidateGeneSearch.jsp).

Associations of *GPR154* haplotypes have been also replicated in different populations. In Laitinen's study, seven haplotypes (H1–H7) were described; H2, H4, H5 and H7 were considered as risk haplotypes for asthma and high total IgE levels and H1, H3 and H6, as non-risk haplotypes [4]. The same haplotypic distribution and a similar pattern of haplotype associations was observed in asthmatic children from five Western European countries and a Swedish population where H5 was significantly associated with asthma and allergic sensitization and H1 was protector for allergic sensitization. However, in this analysis H6 was identified as a risk haplotype for asthma in contrast to the findings in the original study [19]. The haplotypic distribution seems to be preserved in the European populations as observed in Germans and Italians, however, the effect of the haplotypes varied from one population to another. H4 was positively associated with asthma and with asthma accompanied by bronchial hyperresponsiveness [20] in Germans but it was not preferentially transmitted to individuals with elevated total IgE in Italian families [40]. On the contrary, in a Chinese population, although significant associations with airway hyper-responsiveness to methacholine were observed at a single SNP level, a lower haplotypic diversity was described and none of the haplotypes were associated with this phenotype [22].

The relationship among *GPR154* haplotypes and other inflammatory diseases has been also evaluated. For instance, in a study including an Italian, a Swedish and a Finnish case-control datasets, H2 was more frequent in individuals with ulcerative colitis/Crohn's disease in comparison with controls [36]. Moreover, the same risk haplotypes for asthma (H4/H5) were also more frequent in infants with distress respiratory syndrome in comparison with healthy controls and, H1 was also identified as a protector haplotype for this disease in a Finnish population [18]. Since H2, H4, H5 were described as risk haplotypes and H1 as a protecting haplotype in most of the populations, it is possible that these haplotypes contain different alleles of a functional polymorphism or alternatively, this association can be the resulting effect of another variant in linkage disequilibrium in either *GPR154* or another gene.

The SNPs in this region have shown a high pairwise linkage disequilibrium (LD) comprising one or two haplotype blocks in most of the populations analyzed despite different ethnic backgrounds. Differences in LD can affect genotype–phenotype associations making stringent replication more difficult to achieve. In our total sample, we observed a unique 14 kb haplotype block and low *D'* values among some of the SNPs, an LD structure that contrasts with what was observed in other populations, except German [20] and Costa Rican individuals [37]. This finding is interesting, since the Colombian population is an admixed population with European, Native-American

and African ancestries [44] and consequently larger haplotype blocks would be expected. Despite the disparity in the LD pattern, we replicated the association of two *GPR154* SNPs in our population. The haplotypes described here are only partially comparable with those reported by Laitinen et al. and the haplotypic associations observed in the family based study should be interpreted cautiously since the permutation analyses showed lower *P* values than the single analysis in some tests. In our sample, the associations of H4 and H2 with asthma are in the opposite direction from what was observed with haplotypes H3 and H6 in the Finland/Canadian population [5] but are consistent with those described in the European population for childhood asthma [19].

These varying findings emphasize the importance of considering gene-environment effects in association studies of complex diseases such as asthma and allergy sensitization.

The assessment of population structure is critical to interpret appropriately the results of case-control association since stratification and/or differences in the admixture proportions can generate false positive associations in this recently admixed population. The confounding effects given by genetic stratification in our sample can be resolved using measures on individual ancestry obtained from this panel of AIMS (Vergara et al., submitted). This facilitates the reliable use of this sample in population-based genetic association studies. The fact that the associations were consistent in the case control group after controlling for this factor, besides the family dataset, add more consistency to our results.

In general, the epidemiologic evidence justifies a closer look into the functional properties of *GPR154* in the development of asthma, bronchial hyperresponsiveness and atopy. This recently orphanized receptor belongs to the family of G protein coupled receptors, which participates in numerous biologic processes. Recent microarray analyses showed a large *GPR154* downstream gene pathway composed of multiple genes involved in cell proliferation, morphogenesis and immune response including asthma candidate genes like *Tenascin C* (*TNC*) [45], *Prostaglandin-Endoperoxide Synthase 2* (*PTGS2*) and other remodeling genes like *Matrix Metalloproteinase 10* (*MMP10*), *Activin A*, *Interleukin 8* (*IL8*) and *EPH Receptor A2* (*EPHA2*) [46].

An essential role of *GPR154* as participating in the inflammatory process was suggested by the differential expression pattern in airways of asthmatics and controls in the original study [5]. Moreover, in a *GPR154*-deficient murine model, the mutant mice showed an attenuated response to thromboxane (a cholinergic receptor dependent bronchoconstricting agent) which indicates an additional mechanism in asthma, related to its physiologic role as a Neuropeptide S receptor. It was suggested that, since the human airway is highly innervated, the expression of

GPR154 either on sensory or cholinergic neurons would influence the tone of the airway smooth muscle or the response to stimuli [47]. On the other hand, it is well recognized the existing interactions between the neuroendocrine and immune systems mediated via regulation of circulating concentrations of corticosteroid hormones by hypothalamous–pituitary–adrenal axis and numerous reports suggest that the pathogenesis of many chronic inflammatory diseases including atopic dermatitis, rheumatoid arthritis, and asthma can be modulated by stress and emotion. It is possible that polymorphisms in *GPR154* could alter the activity of the central nervous system and consequently impact the risk for the development of asthma. These mechanisms can also give an explanation to the associations found between the polymorphisms and total IgE levels.

In summary, our results highlight once again the role of *GPR154* as a susceptibility gene for asthma and allergies. The risk conferred by this locus is detectable in diverse populations and overcomes the stringency resulting from the differences in the genetic and environmental contexts, the genetic and allelic heterogeneity of the disease and the potential phenotypic variations across the populations.

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