

G protein-coupled receptor 154 gene polymorphism is associated with airway hyperresponsiveness to methacholine in a Chinese population

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Background: A new asthma susceptibility gene, the *G protein-coupled receptor for asthma susceptibility* (GPRA, *GPR154*), has recently been identified and the association was replicated in 2 white populations, but not in a Korean population.

Objective: To test the association between *GPR154* gene polymorphisms and airway responsiveness to methacholine in a Chinese population.

Methods: Eight single nucleotide polymorphisms (SNPs) in the *GPR154* gene were genotyped in 451 cases and 232 controls in stage I. The association of 1 SNP, rs324981, was tested in an additional 264 case and 241 control subjects in stage II. Both single marker and haplotype associations were tested.

Results: In stage I, we found that airway hyperresponsiveness to methacholine was associated with 2 single SNPs, rs324981 and rs324987, but not with the haplotypes of *GPR154*. The minor allele homozygotes of rs324981 (AA) and rs324987 (TT) were at a significantly lower risk of hyperresponsiveness to methacholine with odds ratios of 0.59 ($P = .02$) and 0.56 ($P = .01$), respectively. In stage II, we found a similar trend of association between rs324981 and airway hyperresponsiveness ($P = .09$). In the pooled analysis, the odds ratio of the AA homozygote of rs324981 was 0.61 ($P = .004$). The permutation test resulted in a study-wide empirical P value of .023, which meant that the association remained significant after adjustment for multiple tests.

Conclusions: Our study supports a role of the *GPR154* gene in asthma susceptibility and suggests that the AA homozygote of rs324981 is a protective factor for airway hyperresponsiveness to methacholine in a Chinese population.

Clinical implications: Our findings confirmed a role of *GPR154* in the genetic susceptibility of asthma and suggest that *GPR154* polymorphism should be taken into consideration to improve the assessment of an individual's risk of asthma. (*J Allergy Clin Immunol* 2006;117:612-7.)

Key words: Airway responsiveness to methacholine, asthma, *GPR154*, polymorphism

Laitinen et al¹ performed a genome-wide scan for asthma susceptibility loci in a Finnish population and found a 20-cM region on chromosome 7p14-p15 to be in linkage with asthma and high levels of IgE. By using a hierarchical genotyping design, they identified a 133-kb risk-conferring segment in the linkage region.² Further examination of the segment led to the identification of a novel gene, initially named *GPRA* for *G protein-coupled receptor for asthma susceptibility*, currently referred to as *G protein-coupled receptor 154* (*GPR154*). Laitinen et al² not only identified common *GPR154* haplotypes associated with asthma and high serum IgE in 3 white cohorts but also found that the transcription levels were higher in the bronchi of human beings with asthma and mouse models than in normal controls. This is one of the few asthma susceptibility genes identified by positional cloning in recent years. The association between *GPR154* gene polymorphisms and asthma was confirmed in 2 studies of European children,^{3,4} but not in a Korean population.⁵ The role of *GPR154* in the etiology of asthma in other populations has yet to be examined. In this article, we report the association of *GPR154* polymorphisms with airway responsiveness to methacholine in a Chinese population, using a case-control study design.

METHODS

Study population and phenotype definitions

We conducted a large community-based survey to screen for asthma index families and reference families between 1994 and 1998 in Anqing, a middle-sized city with more than 5 million residents in Anhui Province of China. A detailed description of this population, including sample selection and phenotype measurements, has been presented elsewhere previously.^{6,7} In brief, 2752 index families, which included both parents and at least 2 offspring who were ≥ 8 years old and had physician-diagnosed asthma, were enrolled. In addition, 270 reference families were randomly selected from the same area. The following data were collected from each participant: (1) a questionnaire to assess respiratory symptoms, history of disease, smoking and alcohol consumption, and occupational and environmental exposure; (2) a standardized spirometry test to measure FEV₁ and forced vital capacity (FVC); (3) airway methacholine

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Abbreviations used

FVC: Forced vital capacity
GPR154: G protein-coupled receptor 154
LD: Linkage disequilibrium
OR: Odds ratio
SNP: Single nucleotide polymorphism

challenge test with the following 5 combinations of number of breaths and methacholine concentration in sequential order: 1 breath of 1 mg/mL, 1 breath of 5 mg/mL, 4 breaths of 5 mg/mL, 1 breath of 25 mg/mL, and 4 breaths of 25 mg/mL; the test terminated at the dose that produced a $\geq 20\%$ drop in FEV₁ from the baseline (PD₂₀), or at the final dose if PD₂₀ was not observed; (4) skin prick test; and (5) measurement of serum IgE level and eosinophil count in peripheral blood. We selected 451 cases and 232 controls from the asthma index families in stage I, and an additional 264 cases from the asthma index families and 241 controls from the reference families in stage II, respectively. The cases and controls were selected on the basis of airway responsiveness to methacholine. All of the cases in both stages observed a PD₂₀ on or before the third dose in the methacholine challenge test. All of the controls did not have an observed PD₂₀ during the 5-dose test. One additional criterion was used in the sample selection in stage II but not in stage I: all cases also had self-reported physician-diagnosed asthma and all of the controls did not. All of the cases and the controls in both stages were unrelated to each other within 2 generations—that is, none shared common grandparents. These sample selection criteria and process are presented in Fig 1. The Human Subjects Committees at both the Harvard School of Public Health and the Anhui Medical University approved the study.

Single nucleotide polymorphism selection and genotyping

DNA was extracted from leukocytes in peripheral blood using standard techniques. The investigated single nucleotide polymorphisms (SNPs) were selected from the original association study by Laitinen et al.² From the 13 SNPs they reported to tag the haplotypes, we selected the 10 SNPs that had corresponding reference sequence (rs) numbers in the dbSNP database (<http://ncbi.nih.gov/SNP/>). However, these 10 SNPs were all from intron 2 of *GPR154*. Therefore, we selected 2 additional SNPs in proximity, including a nonsynonymous SNP in exon 3 (rs324981) and a SNP in intron 3 (rs324987), to provide broader coverage of the *GPR154* gene. The 12 selected SNPs were tested in 46 randomly selected Chinese samples using a PCR-RFLP assay. Four out of the 12 tested SNPs were dropped either because they were monomorphic in the test samples or because of PCR failure. The remaining 8 SNPs were all genotyped in our study samples. The SNPs rs323922 (corresponding to SNP522363 in the Laitinen study), rs324377 (SNP529556), rs324396 (SNP563704), and rs740347 (SNP585883) were genotyped by PCR-RFLP. Primer design and restriction enzyme selection were accomplished by using our own software and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The SNPs rs324957 (SNP574953), rs324981 (SNP591694), rs324987 (SNP595009), and rs323920 (SNP520598) were genotyped by using the Taqman genotyping assay designed and manufactured by Applied Biosystems (Foster City, Calif). The number of samples that failed in genotyping was 58 for rs323922, 42 for rs324396, 45 for rs740347, 54 for rs324957, 64 for rs324981, 54 for rs324987, and 64 for rs323920.

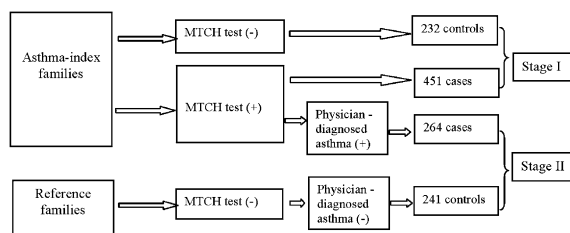


FIG 1. Sample selection process. MTCH, Methacholine.

Statistical analyses

We used a *t* test and a χ^2 test to test for differences in distributions of quantitative traits and categorical variables between cases and controls, respectively. We examined each SNP for Hardy-Weinberg equilibrium in the control group using a χ^2 test. We used Haploview (<http://www.broad.mit.edu/mpg/haploview/>) to calculate the linkage disequilibrium (LD) between the SNPs and to infer the haplotype blocks. We performed single-SNP association tests on methacholine and other asthma related phenotypes using logistic regression or linear regression analyses. We also performed haplotype association tests on hyperresponsiveness to methacholine using Haplo.Score.⁸ To adjust for multiple tests, we performed a permutation test, in which the test statistic is the minimum of all *P* values in the 10 individual tests performed in this study. The distribution of the minimal *P* values under the null was obtained through 1200 permutation cycles. In each cycle, we permuted the case-control status in our samples, performed the same set of association tests described, and obtained the minimum *P* value (*P*_{permute}) among all tests. We estimated the study-wide *P* value as *P* (*P*_{observed} $\geq P_{permute}$). All of these statistical analyses were performed by using the statistical package R (<http://www.r-project.org>).

RESULTS

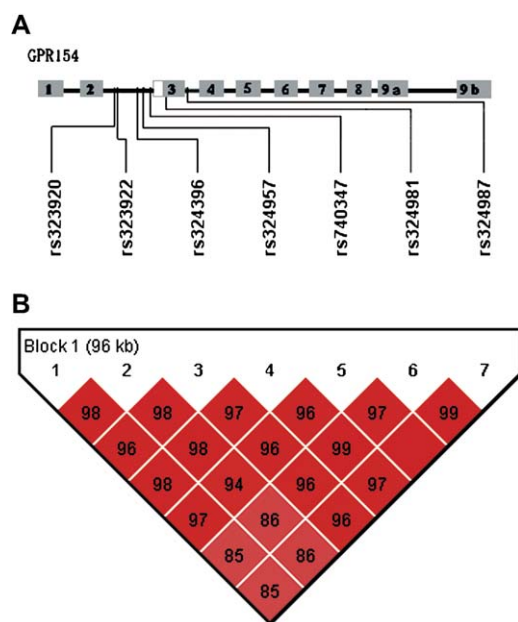
In stage I, we tested the 8 *GPR154* SNPs that form the asthma susceptibility haplotypes in the white population² for association in 683 unrelated subjects (451 cases and 232 controls) selected from our asthma study population. The phenotypic characteristics of the study samples are summarized in Table I. There were no significant differences in age and sex distribution between the cases and the controls. One SNP, rs324377, was removed from analysis because of significant deviation from Hardy-Weinberg equilibrium (*P* < .01). The remaining 7 SNPs were in high LD, with pairwise *D'* values ranging from 0.86 to 1.0 (Fig 2). Two different haplotype block searching algorithms gave rise to slightly different block structures. The 7 SNPs were in a single block using the algorithm based on solid spine of LD. In comparison, only the first 5 SNPs were in the same block using the algorithm of Gabriel et al.⁹ The 7 SNP haplotypes were not associated with airway hyperresponsiveness in both the global and the individual haplotype tests (global *P* = .24; Table II). Three major haplotypes (h1-3) accounted for approximately 90% of the total haplotypes in the samples. Our haplotypes h2 and h3 corresponded to haplotypes H2 and H4, respectively, in the white population,² whereas haplotype h1 could not be mapped

TABLE I. Phenotypic characteristics of the Aqing study population

Characteristic	First study population		Second study population	
	Control (N = 232)	Case (N = 451)	Control (N = 241)	Case (N = 264)
Age (y)	28.4 ± 13.1	30.3 ± 15.6	20.1 ± 11.3	21.3 ± 14.6
Children† (%)	65.9	66.3	53.0	51.7
Male (%)	51.7	53.0	48.1	49.2
Height (m)	1.55 ± 0.13	1.52 ± 0.13*	1.49 ± 0.15	1.42 ± 0.18**
Weight (kg)	49.7 ± 12.8	47.0 ± 13.9*	42.8 ± 13.4	38.1 ± 14.4**
BMI (kg/m ²)	20.2 ± 3.1	19.8 ± 3.3	18.6 ± 3.2	18.0 ± 3.2*
FEV ₁ (L)	2.96 ± 0.85	2.43 ± 0.75**	2.73 ± 0.84	1.96 ± 0.66**
FVC (L)	3.69 ± 1.11	3.49 ± 1.17*	3.19 ± 1.03	2.86 ± 1.16**
Serum total IgE (IU)	425.0 ± 799.1	586.3 ± 1239.2	267.2 ± 474.8	435.4 ± 666.9*
Blood eosinophil count (cell/mm ³)	194.8 ± 241.9	252.0 ± 406.7*	168.5 ± 218.9	302.0 ± 379.4**
Current smoker (%)	33.5	34.2	12.8	21.8
Current alcohol consumer (%)	18.4	14.8	9.8	7.3

P* < .05; *P* < .01.

†Age ≤ 18 y.

**FIG 2. A,** Schematic representation of the 7 SNPs selected for genotyping and analysis, showing their locations on the *GPR154* gene. The numbers in the boxes indicate exon number. **B,** The 96kb haplotype block of *GPR154*, as defined by the solid spine of LD.

to any single haplotype in the previously reported study. We then examined the association between single-SNP genotypes and airway hyperresponsiveness using logistical regression (Table III). Two SNPs, rs324981 and rs324987, were associated with airway responsiveness to methacholine with unadjusted *P* values < .05. The association significance remained the same after model adjustment for age and sex (data not presented). The minor alleles of both SNPs showed a protective effect against airway hyperresponsiveness. These 2 SNPs were in complete LD (*D'* = 1.0). The odds ratios (ORs) among genotypes of these 2 SNPs suggest a multiplicative model (Table III). Among the 7 tested SNPs, rs324981 is the only

nonsynonymous SNP that results in a substitution of Ile with Asn at the 107th codon of *GPR154*. We hypothesized that rs324981 could be a candidate for functional variation in the gene. Thus, we sought to verify this association in additional samples in stage II.

In stage II, we selected from our asthma cohort an additional independent 264 cases and 241 controls. Group phenotypic distributions are also presented in Table I. We found a similar trend of association between rs324981 and airway hyperresponsiveness as seen in the stage I study, although it was not statistically significant (*P* = .09; Table IV). Compared with subjects with the TT genotype at rs324981, ones with the AA genotype had a decreased risk of airway hyperresponsiveness (OR = 0.65; 95% CI, 0.40-1.07). In the pooled analysis, the OR of AA was significantly lower than 1 (OR = 0.61; 95% CI, 0.44-0.85; *P* = .004). The heterozygous AT subjects had a protective trend (OR = 0.86; 95% CI, 0.65-1.13) similar to the AA ones, although it was not statistically significant. Under the multiplicative genetic model, we estimated that each additional copy of allele A reduces the risk of airway hyperresponsiveness by 21% (OR = 0.79; 95% CI, 0.67-0.93; *P* = .006). The stage II statistical significance did not change after model adjustment for age and sex.

Because smoking is related to airway hyperresponsiveness and asthma, we tested the interaction of rs324981 and smoking on airway hyperresponsiveness to methacholine. No statistical significance was found in either separated or pooled analyses.

Overall, we performed 10 association tests on airway hyperresponsiveness, which include 7 single-SNP tests and 1 haplotype test in stage I, 1 single-SNP test in stage II, and 1 pooled single-SNP test. The association between rs324981 and airway hyperresponsiveness was significant, even after adjusting for the 10 tests (study-wide *P* = .023).

In addition, we explored the association of rs324981 with other asthma-related traits, including lung function (FEV₁, FVC, FEV₁/FVC), serum total IgE, eosinophil count in peripheral blood, and skin reactivity to allergens,

TABLE II. Association between the *GPR154* gene haplotypes and airway responsiveness to methacholine in the stage I case-control study

Haplotype	rs323920	rs323922	rs324396	rs324957	rs740347	rs324981	rs324987	Haplotype frequency	P value
h1	T	G	T	A	C	A	T	39.6%	.25
h2	C	C	C	G	G	T	C	37.9%	.30
h3	C	C	C	G	C	T	C	10.3%	.10
Global test									.24

TABLE III. Association between the *GPR154* gene polymorphisms and airway hyperresponsiveness to methacholine in the stage I case-control study

SNP	Genotype	Case N† (%)	Control N† (%)	OR (95% CI)
rs323922	CC	120 (29.6%)	53 (24.1%)	1.0
	CG	201 (49.6%)	109 (49.5%)	0.81 (0.55-1.21)
	GG	84 (20.7%)	58 (26.4%)	0.64 (0.40-1.02)
rs324396	CC	156 (37.0%)	69 (31.5%)	1.0
	CT	189 (44.8%)	104 (47.5%)	0.80 (0.55-1.15)
	TT	77 (18.2%)	46 (21.0%)	0.74 (0.47-1.18)
rs740347	GG	326 (78.4%)	185 (83.3%)	1.0
	GC	81 (19.5%)	34 (15.3%)	1.38 (0.90-2.11)
	CC	9 (2.2%)	3 (1.4%)	
rs324957	GG	153 (37.5%)	68 (30.8%)	1.0
	GA	177 (43.4%)	102 (46.2%)	0.77 (0.53-1.12)
	AA	78 (19.1%)	51 (23.1%)	0.68 (0.43-1.07)
rs324981	TT	140 (35.2%)	62 (28.1%)	1.0
	TA	179 (45.0%)	99 (44.8%)	0.80 (0.54-1.18)
	AA	79 (19.8%)	60 (27.1%)	0.58 (0.37-0.91)*
rs324987	CC	144 (35.4%)	61 (27.5%)	1.0
	CT	184 (45.2%)	101 (45.5%)	0.77 (0.53-1.14)
	TT	79 (19.4%)	60 (27.0%)	0.56 (0.36-0.88)**
rs323920	CC	126 (31.7%)	59 (26.7%)	1.0
	CT	187 (47.0%)	103 (46.6%)	0.85 (0.58-1.26)
	TT	85 (21.4%)	59 (26.7%)	0.68 (0.43-1.06)

* $P = .02$; ** $P = .01$.

†Because of genotyping failure, the total case and control numbers for each SNP may be less than 451 and 232, respectively.

TABLE IV. The association between rs324981 and airway responsiveness to methacholine in the stage II and pooled studies

Study	Genotype	Case N (%)	Control N (%)	OR (95% CI)	P
Stage II	TT	84 (31.8%)	68 (28.2%)	1.0	
	TA	130 (49.2%)	111 (46.1%)	0.95 (0.63-1.43)	.80
	AA	50 (18.9%)	62 (25.7%)	0.65 (0.40-1.07)	.09
Pooled	TT	224 (33.8%)	130 (28.2%)	1.0	
	TA	309 (46.7%)	209 (45.3%)	0.86 (0.65-1.13)	.28
	AA	129 (19.5%)	122 (26.5%)	0.61 (0.44-0.85)	.004

in the pooled samples. We found that the AA genotype was associated with a decrease in FVC ($P < .01$), but not with other asthma-related traits, including serum total IgE (data not presented).

DISCUSSION

Phenotype assessment is critical in genetic studies of complex diseases such as asthma.^{10,11} The clinical

diagnosis of asthma is based on typical characteristics of recurrent episodes of coughing, wheezing, and breathlessness, and lacks standardization. Physicians with different clinical experiences may come up with different diagnoses of asthma. This is especially true in our study population, in which most of the clinical diagnoses were made by local village physicians. An algorithm for the diagnosis of asthma, based on a combination of respiratory symptoms, increased airway responsiveness or bronchodilator response, and a physician's diagnosis of asthma,

has been proposed and applied in our study population.¹¹ However, it remains unclear whether this diagnosis algorithm is appropriate for a genetic study.

Our current association study focused on airway responsiveness to methacholine, a reproducible and objective method associated with the diagnosis of asthma.^{12,13} In addition, we excluded from our association study subjects with an observed >20% drop at the fourth or fifth dosing during the methacholine challenge test. This super case-control design reduces the likelihood of misclassification of the case-control status and has been shown to be more powerful than conventional case-control designs. Kormann et al³ found that SNPs 546333, 585883, and appropriate haplotypes increased the risk for asthma. The associations with these SNPs were even more significant when bronchial hyperresponsiveness was added to the phenotype of physician's diagnosis of asthma. This was also reported in the association of the *ADAM33* gene with asthma.¹⁴ Clear definition of an intermediate phenotype is important in the genetic study of asthma and provides better association than a clinical diagnosis alone.¹⁵ Still, airway hyperresponsiveness to methacholine and clinical diagnosis of asthma have only moderate overlap.¹³ Therefore, our strategy in stage II was to combine response to methacholine with physician diagnosis to ensure selection of cases with asthma and to increase statistical power.

Our results confirm 3 previous association studies of *GPR154* and asthma,²⁻⁴ but differ in the details. Melen et al⁴ genotyped 7 *GPR154* SNPs in 2 European populations. Although 7 haplotypes were identified as the same as in the study by Laitinen et al,² Melen et al⁴ found that haplotypes H1, H5, and H6 were associated with sensitization (high allergen-specific serum IgE), and, furthermore, that H5 was associated with asthma in only 1 of their study populations. In the study by Laitinen et al,² the most common risk-associated haplotypes in the 3 cohorts were H4, H5, H7, and H2. Kormann et al³ genotyped the same 7 SNPs in a large German population. Although the haplotype pattern was very similar, they found that only haplotype H4 (CCAACC) was significantly associated with asthma.

In our study, we selected 8 SNPs, of which 3 (rs323922, rs324396, and rs740347) overlapped with 2 of the previous studies.^{3,4} The haplotype pattern in our Chinese population was different from that in the white populations. There are only 3 major (haplotype frequency >5%) haplotypes in our study population, among which only 2 are the same as in the white populations.² However, we did not find any significant associations between haplotypes and hyperresponsiveness to methacholine. Instead, we found a significant association with rs324981, a nonsynonymous SNP that is very close to the signature haplotype SNPs. Although haplotype analysis has been widely used in association studies, whether it is a more powerful approach than single-SNP analysis in disease gene identification still remains controversial.¹⁶ It's noteworthy that rs324981 is the only nonsynonymous SNP among the 7 SNPs we tested, and ends up being the one with the

most significant association. Unfortunately, the association with rs324981 is not described or examined in the previous studies.²⁻⁵ It would be interesting to examine the association of rs324981 with airway hyperresponsiveness in other populations, and to determine whether this polymorphism elicits changes in the function of the gene product. The haplotype study shows that rs324981 and rs324987, the 2 SNPs that are not selected in previous confirmation studies,³⁻⁵ had weaker LD with rs323920 and rs323922, and were not in the same haplotype block with the other 5 SNPs when using the definition by Gabriel et al⁹ of haplotype block. This could explain why we observed the association only with single markers, and not with the haplotypes. In the stage I study, we found that rs324987 is also associated with airway hyperresponsiveness to methacholine. It is located in the intron of *GPR154*, and is more likely a genetic marker and not a functional polymorphism. Because rs324987 is only 3.3kb from rs324981, and because these 2 SNPs are in high LD, the nonsynonymous SNP rs324981 is more likely the functional gene variation causing asthma. Alternatively, they could both be in LD with a real functional polymorphism nearby. Besides the association with airway responsiveness to methacholine, we also find that the rs324981 polymorphism is associated with the lung function parameter FVC. Although lung function is normal in most new patients with asthma, it deteriorates as the disease progresses.

The case-control design is a common strategy used in genetic studies of complex diseases. Large sample size and population homogeneity are desirable to avoid false negatives and population stratification, respectively. Our study is based on a large sample size (1188 subjects) and is relatively homogenous. All of our study subjects are from a rural area of Anqing, China, a region known for a historically stable population. Not only is there a predominant singular ethnic group, but there exists a uniformity of occupation (farming) and lifestyle throughout Anqing. This population homogeneity minimizes the potential of population stratification that may lead to false-positive associations.

Our study subjects include both children and adults. Although evidence shows that childhood and adult asthma differ in several parameters,^{17,18} the association between the *GPR154* gene and asthma is found in both groups.²⁻⁴ Therefore, the *GPR154* gene may well play a role in the common pathway of childhood and adult asthma.

In conclusion, our study demonstrates that rs324981, a nonsynonymous *GPR154* SNP, is significantly associated with airway responsiveness to methacholine, an objective diagnostic trait of asthma, in a Chinese population. Our study replicates the previously reported association between the *GPR154* gene and asthma in white populations.

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