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Chromosome 7p linkage and GPR154 gene association in Italian families with allergic asthma

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Clinical and Experimental Allergy

Summary

Background Several genome scans have reported linkage of markers on chromosome 7p with asthma and related phenotypes in different populations. A fine mapping in Finnish and French-Canadian populations has associated the GPR154 gene (also known as G-protein-coupled receptor for asthma susceptibility, GPRA) with elevated IgE or asthma.

Objective To confirm chromosome 7p linkage and candidate gene association in Italian families with atopic asthma.

Methods In a two-phase approach, we first performed a linkage analysis of chromosome 7, and then a family-based association study on the GPR154 gene for allergic asthma phenotypes in the Italian population.

Results The screening of 117 families with 19 microsatellite markers showed potential linkage for elevated IgE ($P < 0.002$ at 22 cM from p-ter), asthma ($P < 0.005$ at 44 cM), or atopy ($P < 0.005$ at 54 cM). In the second phase of the present study, candidate gene GPR154, which is located in the phase one-linked region, was investigated in 211 families with seven single nucleotide polymorphisms (SNPs) that tag most haplotype variability, by the pedigree disequilibrium test. Elevated IgE levels were associated with two GPR154 gene SNPs (SNP 546333, $P = 0.0046$; rs740347, $P = 0.006$), and with haplotypes in the global test ($P = 0.013$). Haplotype analysis performed in nuclear families having at least 1 asthmatic parent showed a significant association with asthma ($P = 0.0173$), atopy ($P = 0.0058$), SPT ($P = 0.0025$), and bronchial hyper reactivity ($P = 0.0163$).

Conclusion These results support a susceptibility locus for asthma and related phenotypes on chromosome 7, and are in agreement with recent reports suggesting that a common susceptibility factor for atopic manifestations in asthma is likely conferred by the locus containing the GPR154 gene.

Keywords association, asthma, atopy, GPR154, GPRA, haplotype, linkage, microsatellite marker, PDT, SNP

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Introduction

Several studies have focused on the genetic analysis of asthma and allergy [1], and many locations have been associated with disease, some of which have been confirmed (reviewed in [2–4]).

Linkage of chromosome 7p markers with asthma or related phenotypes has been described in Australian [5], French-Canadian, Finnish [6], and in French asthmatic families [7].

A region of chromosome 7p containing the orphan G protein-coupled receptor gene GPR154 was associated with asthma or elevated IgE levels in Finnish and French-Canadian populations [8]. The GPR154 gene association to asthma or atopy has been more recently confirmed in Swedish, Western European, and Chinese populations [9–11]. The GPR154 gene has been proposed to have functional relevance in modulating asthma by increased expression levels in the relevant tissues and by an inhibitory effect on cell growth [12].

The present study examined Italian families with allergic asthma for linkage to chromosome 7 markers and, after finding potential linkage to chromosome 7p, tested the hypothesis of association with the GPR154 gene.

Methods

Families and phenotypes

Families were collected and phenotyped as described elsewhere [13, 14]. Briefly, 211 families (multiplex, nuclear, or trios) were collected through an allergic asthmatic child. Subjects underwent a clinical evaluation including a methacholine challenge test (BHR). Total serum IgE levels were determined, and skin prick tests (SPTs) for common allergens were performed. The individuals were characterized for clinical asthma through physician diagnosis according to the American Thoracic Society criteria [15], including a positive response to a validated respiratory questionnaire [16]. A subject was considered SPT positive (SPT+) when positive to at least one of the common allergens tested [13]. BHR positivity (BHR+) was defined as a $\geq 20\%$ decline in baseline forced expiratory volume in 1 s (FEV₁) at 25 mg/mL (PC₂₀ < 25 mg/mL). Total serum IgE levels were defined as a dichotomous trait and they were considered to be elevated (IgE⁺) when serum IgE levels were greater than 200 kU/L for individuals over 10-years old, and above the 90th percentile in an age-adjusted standard curve for children under 10. Atopy was defined as the presence of SPT positivity and/or elevated IgE. The protocol was approved by the local ethical committee and informed consent was obtained in the collections of blood and clinical information for all the participants.

Table 1 reports the number of individuals that have been analysed for each phenotype, and the prevalence of each phenotype in the family panel.

Genotyping

In phase one of this study, a chromosome 7 scan was performed by genotyping 117 families with at least one affected sib-pair for one or more phenotypes with DNA microsatellite markers. In phase two, an association analysis was performed by genotyping 211 families (the same 117+94 additional families) with single nucleotide polymorphisms (SNPs). Families in which a marker showed Mendelian inheritance inconsistencies were assigned as untyped for that marker. Individuals in which a marker showed unlikely segregation patterns were recoded as untyped for that marker. The following 19 DNA microsatellite markers were used: D7S1819, D7S2201, D7S1808, D7S817, D7S2846, D7S1818, D7S1816, D7S2204, D7S820, D7S821, D7S1799, D7S1817, GGAA6D03 (also known as D7S1842 or D7S3061), D7S1804, D7S1824, D7S2195,

Table 1. Study subjects phenotyped for asthma and related traits

Phenotype	Individuals		Founders	
	N	Prevalence (%)	N	Prevalence (%)
Asthma	927	35.3	416	12.7
BHR+	825	38.5	394	15.7
IgE+	793	39.7	355	21.9
SPT+	923	56.1	416	38.7
Atopy	927	61.4	417	45.1

Individuals: total number of individuals phenotyped in the sample.

Founders: the individuals without any parent in the family structure.

Prevalence (%): number of individuals/founders with the phenotype on the total number of individuals/founders characterized for the phenotype.

Information on IgE levels was not available in 20 families.

BHR was tested when possible: non-collaborative individuals, individuals with FEV₁ < 70%, or too young (the youngest tested subject was 5.3 years old) were not tested for BHR.

BHR, bronchial hyper reactivity; FEV₁, forced expiratory volume in 1 s.

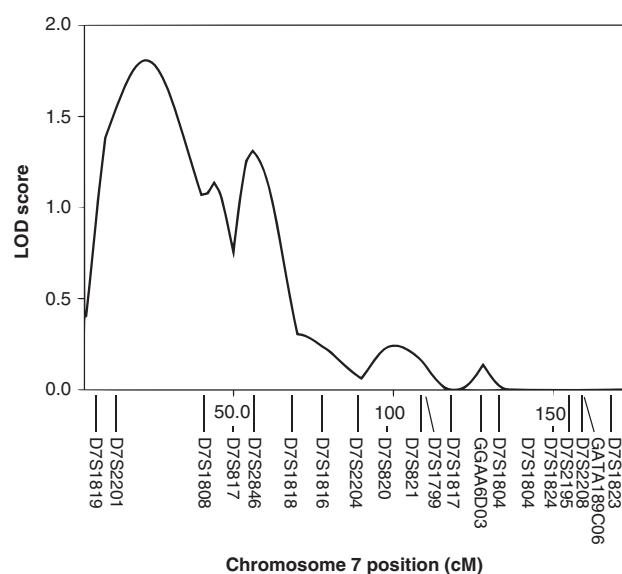


Fig. 1. Multipoint non-parametric linkage analysis of chromosome 7 and elevated IgE.

D7S2208, GATA189C06 (also known as D7S3070), and D7S1823 (Perkin Elmer Europe B.V., Milan, Italy). Fluorescently labelled PCR products were loaded on an ABI377(TM) DNA Sequencer (Perkin Elmer, Foster City, CA, USA) and genotypes were determined as previously described [14]. Two large families (fam #20 and fam #81) that could not be analysed by Merlin program were divided into nuclear families. The newly generated nuclear families were treated as independent. Figure 1 shows the microsatellite markers and their position along the map. The estimated markers' heterozygosity listed in accordance with the order of markers on the map is 0.67,

Table 2. GPRA gene SNPs used for association analyses

			Minor allele	
	SNP	POS_NT_000380	Nucleotide	Frequency
1	rs323917	515224	G	0.07
2	rs323922	522363	C	0.42
3	rs324377	529556	A	0.43
4	SNP 546333	546333	A	0.06
5	rs324384	555608	C	0.44
6	rs324396	563704	T	0.29
7	rs740347	585883	C	0.13

SNPs: from Laitinen et al. [8].

POS_NT_000380: the position of the SNP is given with reference to the public sequence NT_000380.

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&cmd=search&term=NT_000380.

Minor allele frequency was estimated on family founders.

0.66, 0.77, 0.76, 0.76, 0.71, 0.79, 0.88, 0.79, 0.78, 0.73, 0.70, 0.75, 0.83, 0.85, 0.88, 0.76, 0.82, and 0.85. Marker D7S821 showed a significant departure from Hardy-Weinberg equilibrium (HWE, $\chi^2 = 64.9$; $df = 66$; $P = 0.52$; $P < 0.00001$ after permutation test), with a relevant contribution to χ^2 given by a few rare genotype classes, and thus it was removed from subsequent linkage analyses. All other microsatellite markers were in HWE. Microsatellite genotyping was performed in the Section of Biology and Genetics of the Department of Mother and Child and Biology – Genetics of the University of Verona as previously described [14].

The DNA samples were then genotyped for 7 SNPs using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom Inc., San Diego, CA, USA) [17]. PCR assays and associated extension reactions were designed using the SpectroDESIGNER software (Sequenom Inc.) and primer sequences (Metabion GmbH, Planegg-Martinsried, Germany) [9]. Polymorphisms and flanking sequences are described in Laitinen et al. [8]. Table 2 shows the SNPs used, relative position, and allele frequency. The number of samples that failed in genotyping was 10 for rs323917 (1.1%), six for rs323922 (0.7%), 34 for rs324377 (3.8%), 28 for SNP546333 (3.1%), nine for rs324384 (1%), nine for rs324396 (1%), and 11 for rs740347 (1.2%). The number of Mendelian inconsistencies was one for rs323917, four for rs323922, two for rs324377, three for SNP546333, three for rs324384, 0 for rs324396, and four for rs740347. The families showing Mendelian inconsistencies were set to ungenotyped for the marker showing the inconsistencies.

SNP rs740347 showed a significant departure from HWE in founders ($P = 0.008$) but not in the total sample set. The slight departure in the only subgroup of founders was attributed to random sampling effects rather than genotyping difficulties. Therefore, SNP rs740347 was retained in the SNP panel.

SNP genotyping was performed in the Clinical Research Centre, Karolinska Institute, as previously described [9].

Statistical analyses

Multipoint non-parametric linkage analysis was performed with the computer program Merlin [18], and the 'NPL all' statistics [19] were used. The logarithm of odds ratio (LOD) score and the statistical significance (P -value) were estimated according to Kong and Cox [20]. The family-based association study was performed by the pedigree disequilibrium test (PDT) [21, 22] that includes uncertain haplotypes in general pedigrees. This is an implementation of the pedigree disequilibrium test with extensions to deal with haplotypes and missing data [23]. The global test for all haplotypes is obtained by the marginal homogeneity statistic [24].

Haplotypes were estimated by the determination of marker segregation within the families with the programs Merlin or PDT. Merlin reconstructed haplotypes for families and individuals by the most likely pattern of gene flow (best option). Haplotype frequency was then calculated from the parents with unambiguous haplotypes. The eight haplotypes with an estimated frequency $> 2\%$ are given in Table 3.

The PDT analysis was performed on all the families or on the nuclear families having at least one asthmatic parent. Nuclear families were generated by splitting the multiplex families into nuclear families (sibling and parents). No more than one nuclear family was from the same multiplex family.

The PDT haplotype analysis has been computed on 211 families (five phenotypes), and in a subset of 59 families (five phenotypes), for a total 10 (5+5) tests. Applying the conservative Bonferroni's correction for multiple tests, a single test would be significant at the 0.05 nominal level, after 10 independent tests, when the corresponding P -value was $P = 0.0051$. This overall P -value is actually smaller than the nominal 0.05 and should be corrected by an unknown factor that depends on the lack of independence among tests.

A permutation test was used to estimate the significance of association in the tests that showed a significant association when all families were included in the analysis.

Computer program

The following computer programs were used: Merlin (available from <http://www.sph.umich.edu/csg/abecasis/Merlin/>); Package Unphased (available from <http://www.mrc-bsu.cam.ac.uk/personal/frankhgmmp.mrc.ac.uk/~fdudbrid/software/unphased/>); R package (available from <http://www.R-project.org>, <http://www.r-project.org/>) [25]; and the haplo.em provided by the haplo.stats library

Table 3. Haplotypes and haplotype analysis for elevated IgE in the whole sample

Haplotype	PDT and IgE			GPR154 haplotypes						
	%	Z-score	P	rs323917 [C/G]	rs323922 [G/C]	rs324377 [C/A]	SNP 546333 [G/A]	rs324384 [T/C]	rs324396 [C/T]	rs740347 [G/C]
H1	0.2837	0.6686	0.5038	C	G	C	G	C	T	G
H2	0.2108	1.118	0.2634	C	C	A	G	T	C	G
H3	0.1506	0.4957	0.6201	C	G	C	G	C	C	G
H4	0.0539	-3.014	0.002582	C	C	A	A	T	C	C
H5	0.0729	-1.271	0.2038	C	C	A	G	T	C	C
H6	0.1189	-0.311	0.7558	C	G	C	G	T	C	G
H7	0.0650	0.4343	0.664	G	C	A	G	T	C	G
H8*	0.0238	-1.944	0.05191	C	G	A	G	T	C	G

Global test χ^2 : 14.62. Empirical significance: $P=0.041$.

Global significance: 0.013 SE: 0.003 (1000 permutations).

The table shows the transmission of the haplotypes to individuals with elevated IgE (output from pdtphase computer program of UNPHASED package). PDT was launched with the following flags: -rare 0.02 -missing, -observed, -droprare, -EM (pdtphase computer program).

The haplotypes were reconstructed according to the algorithm implemented in the program Merlin. Individuals with ambiguous haplotypes were not counted. Haplotypes are named according to Laitinen et al. [8]. Only haplotypes with estimated frequency > 2% are shown.

*H8 haplotype was described by Melen et al. [9] and Veal et al. [30], but was not reported in Finnish and French-Canadian samples. The frequency was estimated from family founders.

(<http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm>).

Several scripts were developed for automatic analysis, arrangement of the output, and estimation of significance of deviation from HWE by the permutation test.

Results

Asthmatic Italian families were studied first for linkage to chromosome 7, and then for association to the GPR154 gene.

Linkage to chromosome 7

One hundred and seventeen families were genotyped with 19 microsatellite markers (Table). Non-parametric linkage analysis showed the following nominally significant linkages on the chromosome map: elevated IgE at 22 cM (LOD = 1.81; $P=0.002$), clinical asthma at 44 cM (LOD = 1.48; $P=0.005$), and for atopy at 54 cM (LOD = 1.43; $P=0.005$). Figure 1 shows the LOD score plot for elevated IgE across the marker map. Because we tested the hypothesis of linkage based on an *a priori* observation, the linkage results confirmed the significant linkage of this locus.

Association to the GPR154 gene

The GPR154 candidate gene is located between microsatellite markers D7S817 and D7S2846. Seven SNPs located in the GPR154 gene (table 2) were used for an association study in 211 families.

The PDT showed that two SNPs were associated with elevated IgE levels: SNP 546333 ($P=0.0046$) and

rs740347 ($P=0.006$). No other association was observed for any of the seven SNPs with any other phenotype.

The haplotype transmission analysis showed a global significant association for the elevated IgE phenotype only. No significant association was observed for any of the other phenotypes.

Table 3 shows that the H4 haplotype was preferentially not transmitted to individuals with elevated IgE (Z-score = -3.014; $P=0.0025$; global test $\chi^2=14.2$ P -value = 0.041). The permutation test shows a global significance ($P=0.013$).

The PDT analysis was also performed in the subset of 59 nuclear families having at least one asthmatic parent. Haplotype analysis showed a significant association with asthma (global test $\chi^2=15.4$, $P=0.0173$; detailed in table 4), atopy (global test $\chi^2=18.19$, $P=0.0058$), SPT (global test $\chi^2=20.2$, $P=0.0025$), and BHR (global test $\chi^2=15.56$, $P=0.0163$). No significant association was observed for the IgE phenotype. Haplotype H6 was observed to be preferentially transmitted to affected individuals, and haplotypes H4 and/or H5 were observed to be preferentially not transmitted to affected individuals.

Discussion

Several genome scans for asthma have a reported linkage on chromosome 7p [5–7]. In the first phase of the present study, we screened chromosome 7 with 19 DNA markers on 117 Italian families having two or more affected sibs for at least one of the following phenotypes: asthma, BHR, elevated IgE, SPT, or atopy. Potential linkage was observed at different positions from 22 to 54 cM in the marker map, for asthma ($P < 0.005$ at 44 cM), elevated IgE

Table 4. Haplotype analysis for asthma in nuclear families with at least an asthmatic parent.

Haplotypes	Z-score	P
H1	1.413	0.1577
H2	0.08472	0.9325
H3	-0.5085	0.6111
H4	-1.812	0.06991
H5	-2.23	0.02573
H6	2.657	0.007889
H7	-0.6255	0.5316

Global test χ^2 : 15.4; $P = 0.01734$.

Haplotypes are coded as in Table 3.

($P < 0.002$ at 22 cM), and atopy ($P < 0.005$ at 54 cM). These results confirm previous linkage observations on chromosome 7p.

In 2001, a genome scan in three founder populations [6] reported linkage of chromosome 7p with asthma and related phenotypes (elevated IgE, atopy). The linked region spanned about 20 cM of the chromosome. Previously, in 1996, Daniels et al. [5] had described linkage of chromosome 7p markers with IgE levels in asthmatic Australian families. We performed the screening of chromosome 7p to investigate whether it may contain a susceptibility gene for asthma phenotypes in the Italian population. The chromosome 7 screening has been performed with 19 DNA markers on 117 families having two or more affected sibs for at least one of the asthma phenotypes (asthma, BHR, elevated IgE, SPT, atopy). The screening showed potential linkage at different positions ranging from 22 to 54 cM in the marker map, for asthma ($P < 0.005$ at 44 cM), elevated IgE ($P < 0.002$ at 22 cM), and atopy ($P < 0.005$ at 54 cM). This linkage result suggests that a genetic susceptibility factor may map on chromosome 7p, and enhances the possibility that the linkage peak observed in Finnish, French-Canadian, Australian, and now also in the Italian families, may be due to the same genetic factor.

In the second phase of the present study, the recently described GPR154 gene was analysed under the hypothesis that it might represent a likely candidate. The PDT was performed on 211 families (the same 117 families used for chromosome 7 screening +94 with at least one asthmatic child). Single marker analyses showed an association of the elevated IgE phenotype with two SNPs (SNP 546333, $P = 0.0046$; rs740347, $P = 0.006$). The seven SNPs accounted for eight frequent haplotypes and the PDT showed a significant global haplotype transmission disequilibrium ($P = 0.041$; $P = 0.013$ after 1000 permutations) with the elevated IgE phenotype. The selection of nuclear families having at least one asthmatic parent may help to target families with an at-risk segregating gene. The PDT haplotype analysis was also performed in the 59 nuclear families and it showed a significant association

with asthma, SPT, and atopy, and indicated that haplotype H6 is preferentially transmitted to affected individuals.

The seven SNPs were selected because they tag the most common haplotypes for asthma susceptibility association to a 133 Kb long GPR154 gene region in the Finnish population. Laitinen et al. [8] identified four risk haplotypes (H2, H4, H5, H7) for either elevated IgE or asthma phenotypes in the Finnish and the French-Canadian populations. This observation was partially replicated in recent studies by Kormann et al. [10], and by Melen et al. [9], in asthmatics from the German, Swedish, and other Western European populations, respectively, while in the Bamse cohort of Swedish children alone no significant association was observed [9]. Recently, the GPR154 gene association with BHR in asthma has been described in a Chinese rural population with a coding polymorphism (rs324987, allele T) [11]. This allele tags the haplotypes H2, H4 or H5, H6, and H7 in Caucasian individuals (inferred from the HAPMAP database [26]).

The association with the GPR154 gene was not replicated in the Korean population [27]; the lack of replication may be due to a different genetic and environmental background between Korean and European populations, or due to the incomplete genotyping using only one SNP and no haplotype analysis in the Korean sample.

The study in German asthmatic children provided evidence of association with asthma and bronchial hyper-responsiveness with haplotype H4, and with a lower degree with total serum elevated IgE [9, 10].

It is intriguing that H4 and H5, which differ in only one SNP, are likely risk haplotypes in other populations [8–10], and that they have been preferentially not transmitted to affected children in the present study (table and Table 4). The reduced transmission of H4/H5 haplotypes could be taken to indicate that they are not risk haplotypes in the Italian sample. However, the transmission disequilibrium tests we used are different from the classical case-control studies used in the other population, in which the controls are unaffected individuals. The controls in the transmission disequilibrium tests are represented by the untransmitted alleles that cannot easily be associated to affected or unaffected status. Therefore, any hypothesis on the involvement of untransmitted alleles should consider that controls are not unaffected entities.

The PDT analysis in 59 nuclear families with asthmatic parents showed that H6 is preferentially transmitted to the affected individuals.

The overtransmission of H6 haplotype might have affected the observed pattern of H4/H5 haplotype segregation. This might either be due to a major involvement of the H6 haplotype with allergic asthma in the Italian population sample or to a random access of H6 transmission with respect to other susceptibility haplotypes (i.e. H4, H5).

Table 5. GPRA haplotypes associated with asthma or related phenotypes in the published studies

Haplotype	Phenotype	Risk	Population	P-value
H1	Asthma	—	3	0.054
	Sensitization	—	4, 4+5	0.014, 0.038
	Allergic Asthma	—	4+5	0.045
H2	Asthma	+	2	0.0009
	IgE		1	0.051
H3	None	None	None	NS
H4	Asthma	+	3	0.024
	Asthma+BHR	+	3	0.036
H5	Asthma	+	4	0.033
	Sensitization	+	4, 4+5	0.033, 0.019
	Allergic Asthma	+	4+5	0.023
H6	Sensitization	+	4, 4+5	0.017, 0.015
	Allergic Asthma	+	4+5	0.023
H7	none	None	None	NS
H4, H5	IgE	+	1	0.010
H(2,4,5,7,n) vs	Asthma	None	6	NS
H(1,3,6)	Asthma, IgE	None	7	NS
—	Asthma, IgE	none	8	NS
H(2,4)	BHR	+	9	NS

1, Finnish [8]; 2, French-Canadian [8]; 3, German [10]; 4, Parsifal [9]; 5, Bamse [9]; 6, Korean [27]; 7, asthma or IgE in patients ascertained for atopic dermatitis (German, Italian, Swedish and the Netherlands population) [31]; 8, asthma or IgE in patients ascertained for atopic dermatitis (Northern European population) [30]; 9, Anqing, China.

NS, significant association was observed for individual SNP but not for haplotypes.

Of the 10 PDT tests performed, the IgE phenotype was significantly correlated in the set of 211 families, and asthma, SPT, atopy, and BHR in the subset of nuclear families with at least one asthmatic parent. Therefore, the number of positive tests exceeds the expected under a random effect (i.e. the probability to observe five significant tests out of 10 is $P=0.00006$) and applying the conservative Bonferroni correction, one test showed a significant association with SPT in the 59 nuclear families.

Table 5 gives a summary of GPR154 haplotype association to asthma-related phenotypes in the published studies. The present data therefore confirm that, in a Southern European population sample, markers on 7p and GPR154 haplotypes are associated with elevated IgE levels, a phenotype related to allergic asthma.

The genomic segment harboured two novel genes: GPR154 and AAA1 (asthma-associated alternatively spliced gene 1): while AAA1 may not represent a protein-coding gene, GPR154 showed a distinct distribution of protein isoforms in bronchial biopsies from asthmatics individuals, and its murine orthologue was up-regulated in a mouse model of ovalbumin-induced inflammation [8]. GPR154 has two main full-length isoforms, named GPRA-A and GPRA-B; the activation of GPRA-A isoform by its agonist, neuropeptide S (NPS), results in an inhibi-

tion of cell growth *in vitro*, while the GPRA-B did not show NPS-mediated activation, and therefore its role in asthma pathogenesis is still unknown [12].

Recently, the NOD1 gene, which is located about 3.5 Mb from the GPR154 gene, has been associated with asthma in Australian and European populations [28, 29]. It is unlikely that linkage disequilibrium extends over 3.5 Mb, confounding the associations of the GPR154 gene with the NOD1 gene.

In conclusion, the present study suggests that a common susceptibility factor for atopic manifestations in asthma maps within, or close to, the GPR154 gene in Italian families. The variation in haplotype associations in different populations, age groups, and environmental exposure groups [8–10] may suggest that the effects of the susceptibility locus interact with environmental factors, and future studies should be targeted to resolve such gene–environment interactions.

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Disclosure of potential conflict of interest

The authors have disclosed no conflict of interest.

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