

Chromosome 14 linkage analysis and mutation study of 2 serpin genes in allergic asthmatic families

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Background: Genome and chromosome screens reported DNA markers on chromosome 14 linked to allergic asthma or intermediate phenotypes in several populations.

Objective: We sought to perform a linkage study on chromosome 14 and a further association study on candidate genes mapped in the region found to be linked to allergic asthma or intermediate phenotypes.

Methods: The study consisted of a sample of 189 families (847 genotyped individuals) from a restricted geographic area in northeastern Italy. The subjects were characterized for the following phenotypes: allergic asthma, total serum IgE levels, skin prick test responses, and bronchial hyperresponsiveness (BHR) to methacholine. Genotyping was done with 14 DNA markers and 4 polymorphisms in the genes encoding α_1 -antitrypsin and α_1 -antichymotrypsin (ACT).

Results: Multipoint analysis indicated a potential linkage of BHR with marker D14S617 (nonparametric linkage z score = 2.32, $P = .01$). Transmission disequilibrium of Thr -15Ala in the gene encoding ACT was observed with all the phenotypes investigated: allergic asthma, BHR, total IgE levels, or skin prick test responses ($P = .041$, $.02$, $.0053$, or $.026$, respectively). **Conclusion:** Chromosome 14 screening and transmission disequilibrium testing on the gene encoding ACT suggest that it or a closely located gene may be involved in susceptibility to allergic asthma in the Italian population. (J Allergy Clin Immunol 2001;107:654-8.)

Key words: α_1 -Antichymotrypsin, α_1 -antitrypsin, allergic asthma, bronchial hyperresponsiveness, transmission disequilibrium test, chromosome 14, linkage, association

The inheritance of asthma and allergy does not follow the classical Mendelian patterns that are characteristic of single-gene disorders.¹ Many genetic studies reported linkage or association of allergic airway inflammation

Abbreviations used

ACT: α_1 -Antichymotrypsin

AT: α_1 -Antitrypsin

BHR: Bronchial hyperresponsiveness

NPL-Z: Nonparametric linkage z score

SPT: Skin prick test

TDT: Transmission disequilibrium test

diseases to several chromosomal regions. Also, polymorphisms in a few candidate genes indicated that a genetic component could be involved in the etiopathogenesis of the disease in different populations.² In particular, suggestive evidence for genetic linkage between atopic asthma and chromosome 14q markers has been reported in UK,^{3,4} Australian,⁴ white US,⁵ and Hutterite⁶ populations, respectively.

The serine protease inhibitor (serpin) genes map on chromosome 14 and have been cloned.⁷ They may be involved in the development of chronic obstructive pulmonary diseases, as suggested by several studies.⁸⁻¹⁰ A possible association between asthma and α_1 -antichymotrypsin (ACT) or α_1 -antitrypsin (AT) deficiency, respectively, has been reported.^{11,12} Moreover, a correlation between airway hyperresponsiveness and AT S mutation carriers has been suggested.¹³

In this study we have investigated the presence of susceptibility loci on chromosome 14 for phenotypes related to allergic asthma in a sample of the Italian population, and we have further performed an association study in candidate serpin genes that map in the region where chromosome screening suggested linkage.

METHODS

Subjects

A panel of 847 subjects belonging to 189 families from northeastern Italy was recruited through an allergic asthmatic child attending the Department of Mother and Child of the University of Verona or the Hospital of Bolzano. The allergic asthmatic child was defined as having skin prick test (SPT) positivity and asthma. Mean age of the probands was 9 years (range, 2-19 years), and that of the other participants was 29.1 years (range, 1-82 years). The protocol was approved by the local ethical committee. Data for the following phenotypes were collected: bronchial hyperresponsiveness (BHR) to methacholine (756 individuals); total serum IgE level (731 individu-

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TABLE I. Distribution of phenotype among asthmatic and nonasthmatic subjects

	BHR		SPT		IgE	
	Yes	No	Yes	No	Yes	No
Asthma						
Yes	234 (79.6%)	60 (20.4%)	292 (94.5%)	17 (5.5%)	203 (74.6%)	69 (25.4%)
No	68 (14.8%)	393 (85.2%)	181 (34.2%)	349 (65.8%)	94 (20.6%)	362 (79.4%)

BHR, Response at PC₂₀ values of less than 25 mg/mL; SPT, response to one or more common allergens; IgE, total serum elevated IgE.

als); SPT reactivity against common aeroallergens (844 individuals); and asthma (847 individuals). Asthma was defined according to the American Thoracic Society criteria,¹⁴ including positive response to a validated respiratory questionnaire (International Study of Asthma and Allergies in Childhood).¹⁵ BHR was defined as a fall in baseline FEV₁ by at least 20% at 25 mg/mL (PC₂₀ < 25 mg/mL).¹⁶ Subjects with FEV₁ of less than 70% (8 individuals) were considered to have positive evidence of BHR, even if they did not undergo methacholine challenge. A total of 27 common standardized allergens, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Felis domesticus* (cat), *Canis familiaris* (dog), Gramineae, and a mixture of allergens of trees, epithelia, and grass, were tested. The test response was considered positive when the diameter of the wheal was 3 mm larger than that of the saline control. A subject was considered to have positive SPT responses when he or she had positive results to at least one allergen. Elevated IgE were defined as a qualitative trait with serum concentration greater than 200 kU/L for adults and above the 90th percentile in an age-adjusted standard curve value for children under 10 years. The mean total serum log IgE level in the probands was 2.68 ± 0.47 (range, 1.38-3.91) and 1.88 ± 0.65 (range, 0.30-3.89) in the other participants. Preschool children were considered asthmatic if they had the presence of more than 3 wheezing lower respiratory tract manifestations in the previous 6 months, wheezing apart from colds, presence of sensitization to indoor allergen, and/or positive response to inhaled corticosteroid treatment, β_2 -agonists, or both.^{17,18} Table I shows the distribution of the phenotypes in asthmatic and nonasthmatic individuals.

Chromosome screening

Genomic DNA was extracted from whole blood with standard methods. Fourteen microsatellite DNA markers and 4 single nucleotide polymorphisms in the genes encoding AT and ACT were used. In the chromosome screening of all affected sib pairs containing families, the following markers were used: D14S742, D14S1280, D14S608, D14S306, D14S583, D14S587, D14S588, D14S606, D14S616, D14S617, D14S1434, D14S605, D14S614, and D14S1426 (Research Genetics Inc, Huntsville, Ala). The sex-averaged spacing was 9.8 cM, with a maximum estimated distance between markers of 18.8 cM. A limited number of families (n = 35) was previously genotyped also for marker D14S63 (data not published). Genotypes were determined by using the ABI-PE 377 Nucleic Acid Analyser (PE Applied BioSystems, Foster City, Calif) after PCR with fluorescent primers.

Serpin genes mutation analysis

All subjects were genotyped for the following 3 mutations in the gene encoding AT: Glu264Val(S), Glu342Lys (Z), and G1237A in the 3' flanking sequence. Subjects were also genotyped for one mutation in the gene encoding ACT: Thr -15Ala in the signal peptide. These mutations were determined by using PCR and restriction analysis with the appropriate endonucleases, as recently described.⁹

Statistical analysis

IgE levels were treated as both a quantitative trait and a qualitative

TABLE II. Families collected and nuclear families after splitting

	Families (inclusive)	Families (nuclear)
	172	1
	10	2
	4	3
	1	4
	1	5
	1	11
Total	189	224

Families (inclusive), as collected; families (nuclear), 2-generation families only.

trait; all the other phenotypes were treated as qualitative traits. Genetic analysis was performed with nonparametric linkage procedures and the transmission disequilibrium test (TDT).¹⁹ The following computer programs have been used: Genehunter 2 release 2.20 and program sib_tdt implemented in the ASPEX package.^{21,22} A chromosome 14 reference map was built from 15 Centre d'Etude du Polymorphisme Humain families by using the computer program CRIMAP 2.4.²³ The order of the markers and their estimated distances are reported in Fig 1. The marker order is in agreement with the one reported in the genetic location database (http://cedar.genetics.soton.ac.uk/public_html/ldb.html). The map built from our sample shows the best support for the same estimated marker order. TDT is a generally valid χ^2 test for association only if transmissions to unrelated affected children are used in the analysis.²⁴ All families with affected sib pairs were used for linkage analysis. For TDT analysis, families with only one affected child were also used, and multiplex families (n = 17) were split (n = 52) into a total of 224 nuclear families. Table II shows the number of families of the sample before and after splitting. Table III gives the number of affected children for each phenotype investigated per nuclear family. The nuclear families were treated as independent, and genotypes were not imputed.

We performed the TDT after 2 separate procedures. First, we randomly selected one affected child from each sibship and applied the TDT. This method uses a limited amount of available information, and the result depends on the sample randomly selected. The following number of nuclear families with one affected child was selected: 205 families for asthma, 174 for BHR, 152 for IgE, and 206 for SPT. Second, the sib_tdt program was used, which calculates empirical probabilities for χ^2 statistics and which accurately reflects association independent of linkage within families by permuting parent alleles while fixing the identity-by-descent (IBD) status of sibs within a family.²² This allows us to use the information from all the affected sibships.

RESULTS

The study was carried out in 2 stages: (1) chromosome screening and (2) association study in candidate genes that map in the chromosomal region positive to linkage analysis.

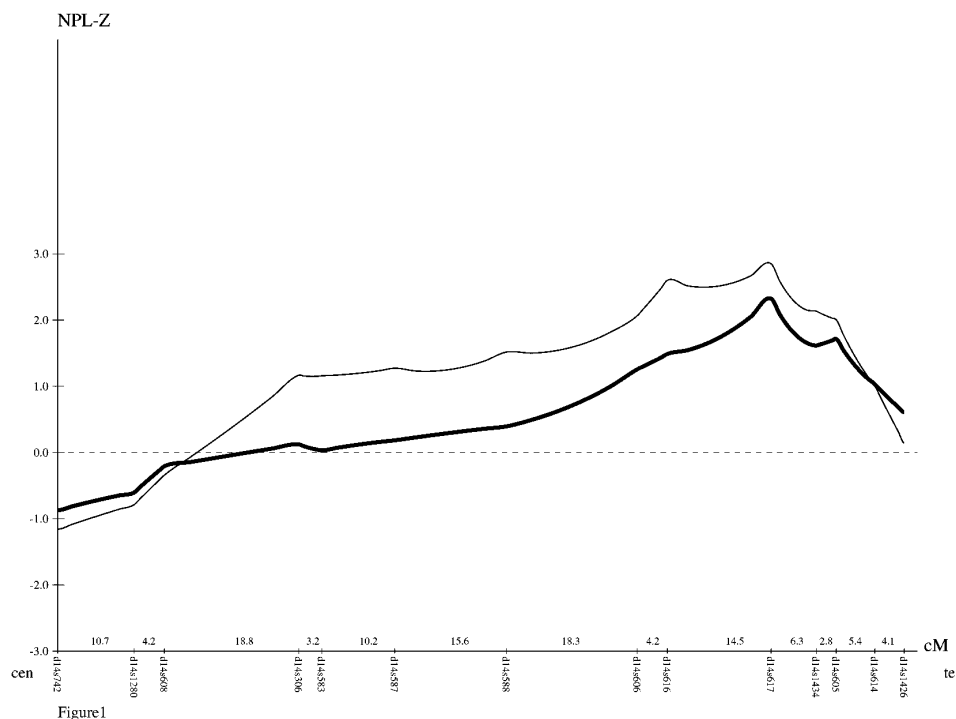


FIG 1. Multipoint linkage analysis between chromosome 14 markers and BHR. Linkage results (NPL-Z on y-axis) of nonparametric multipoint linkage analysis for BHR PC₂₀ values of less than 25 mg/mL (*heavy line*) or for BHR PC₂₀ values of less than 10 mg/mL (*light line*). On the x-axis, DNA markers and their estimated distances (in centi-Morgans) are reported.

TABLE III. Distribution of affected children in nuclear families

No. of affected children	No. of nuclear families			
	Asthma	BHR	IgE	SPT
0	18	49	71	17
1	154	111	92	109
2	44	59	49	76
3	7	4	10	16
4	1	1	2	4
5	0	0	0	1
6	0	0	0	1

Chromosome 14 screening

All traits (allergic asthma, BHR, IgE, and SPT) were investigated for linkage to the chromosome 14 markers in all families with affected sib pairs (109 families and 538 typed individuals). We observed a potential linkage for BHR, as shown in Fig 1. BHR showed a multipoint nonparametric z score (NPL-Z) equal to 2.34 ($P = .01$) around marker D14S617. BHR, more strictly defined as a PC₂₀ value of less than 10 mg/mL, was again linked to D14S617 with a higher significance value (NPL-Z = 2.87, $P = .002$, Fig 1). Asthma showed an NPL-Z of 1.73 ($P = .042$) for D14S617. No other marker for any other phenotype studied showed NPL-Z with associated P val-

ues of less than .05. Quantitative analysis on log IgE did not suggest linkage of IgE levels to any marker. The analysis on 35 families of marker D14S63 reported negative NPL-Z scores.

Serpin genes

Four mutations in the 2 serpin genes were investigated in all the subjects: S, Z, and G1237A in the gene encoding AT and Thr -15Ala in the gene encoding ACT, respectively. The frequency of the alleles was calculated on the founders, and it was as follows. For the gene encoding AT, S is 0.009 ± 0.004 ($\pm$ SD), Z is 0.007 ± 0.003 , and 1237A is 0.13 ± 0.01 , and for the gene encoding ACT, Thr is 0.47 ± 0.02 .

Because of the low frequency of mutation Z, the TDT was not performed. However, there was no indication of a preferential transmission of mutation Z to the affected individuals (ie, 7 transmissions for SPT: 2 transmitted and 5 not transmitted).

Mutation S was found to be transmitted 7 of 11 times to individuals with positive SPT responses ($P =$ not significant), and the low number of observations does not allow us to draw conclusions. No significant transmission disequilibrium was observed for mutation 1237A/G for any phenotype investigated (eg, 51 transmitted and 52 nontransmitted for SPT phenotype).

The -15Ala allele of the gene encoding ACT was preferentially transmitted to the affected individuals with all

phenotypes: asthma ($P = .041$), BHR ($P = .02$), elevated IgE ($P = .0053$), and SPT ($P = .026$; Table IV). No preferential maternal or paternal allele transmission was observed for mutation ACT -15Ala. We observed similar results by randomly selecting one affected individual from each family (data not shown). We repeated the above random selection procedure 1000 times for IgE, and 530 samples showed an excess of -15Ala transmission ($P < .05$). The remaining 470 samples did show an increased transmission of -15Ala to the affected individual, even if not significant.

More detailed linkage and association results are reported at <http://medgen.univr.it/asthma>.

Linkage of the D14S617 marker and transmission disequilibrium of the ACT -15Ala allele analyses were also performed, dropping the individuals with FEV₁ of less than 70%. There was no difference in the significance of the results (data not shown).

DISCUSSION

We describe a potential linkage of the D14S617 (14q23-q32) marker to BHR and a suggestive association of allele -15Ala of ACT in the serpin gene cluster to allergic asthma phenotypes. This is the first report of such a linkage in any population and the first report of the serpin gene cluster association in allergic asthmatic families.

We screened chromosome 14 with DNA markers after some suggestions reporting chromosome 14 regions or genes associated to allergic asthma phenotypes. The T-cell receptor α/δ cluster (14q11) has been reported to be linked to an elevated total IgE level or specific IgE in British and Australian populations.^{3,4} The Collaborative Study on the Genetics of Asthma reported markers linked to asthma in the region of T-cell receptor α/δ in white US subjects and in Hutterites (<http://www.csga.org> and <http://genes.bsd.uchicago.edu/hutterite>).^{5,25} We did not find any indication of linkage to this chromosomal region.

Two genome scans reported possible linkage in the same region we are reporting for phenotypes associated to pulmonary respiratory function. The German genome scan reported linkage of peak expiratory flow rate values to marker D14S977 (asthma gene database <http://cooke.gsf.de>).²⁶ The genome scan for atopy in British and Australian population reported linkage of marker D14S51 and wheezing and whistling (<http://www.well.ox.ac.uk/asthma/public/GenomeScan/results.htm>).²⁷ In these studies there are weak suggestions of linkage that do not follow the more strict criteria of linkage given by Lander and Kruglyak.²⁸ Even if type I error can cause the finding of linkage by chance, it is suggestive that the 3 screens, including the present report, reported positive results in the same chromosomal region. In this region serpin genes as well as other genes are located which could contribute to chronic pulmonary respiratory diseases in the German, British, Australian, and Italian populations. The observation of a wide linkage (Fig 1) makes it difficult to define with precision the chromosomal region and the candidate genes.

TABLE IV. Transmission disequilibrium tests between -15Ala allele and phenotypes investigated in families with atopic asthmatic children

Phenotype	T	NT	χ^2	P value*
Asthma	129	97	4.53	.041
BHR	117	83	5.78	.02
IgE	118	78	8.16	.0053
SPT	161	121	5.67	.026

T, Number of alleles transmitted to affected children; NT, number of alleles not transmitted to affected children; BHR, response at PC₂₀ values of less than 25 mg/mL; SPT, response to one or more common allergens; IgE, total serum elevated IgE.

*P values were determined by using a permutation test (sib_tdt program).²²

The serpins are a family of protease inhibitors that play a central role in the control of the proteolytic cascade.²⁹ These genes have been suggested by the chromosome screening as positional candidates. The serpin gene cluster maps within 1.5 Mb (Genetic Location Database) from the D14S617 marker. In the Centre d'Etude du Polymorphisme Humain genotype database (<http://www.cephb.fr/cephdb/>) the 2-point distance between the D14S617 marker and the genes encoding ACT or AT is 4 cM (CI, 0-12) and 7 cM (CI, 3-13), respectively.

The allele -15Ala of antichymotrypsin gene in the signal peptide was found to be preferentially transmitted to the affected subjects in this study. ACT could be involved in airway remodeling in allergic asthma according to the protease-antiprotease hypothesis,³⁰ perhaps with an effect on elastase fragmentation and thickening.³¹ The mutation Thr -15Ala is not related to the level of ACT.¹⁰ The mutation was described to occur in German patients with chronic obstructive pulmonary disease and control subjects at a similar frequency.³² The frequency of the mutation reported in that study is different from the frequencies estimated in our study from family founder members (53%) or from a sample of Italian control individuals (47.9%).⁹ This indicates that the mutation might have a different frequency between German and Italian populations. Recently, the same allele has been associated with chronic obstructive pulmonary disease in the Japanese population.¹⁰ The authors hypothesize that the Thr -15Ala genotype was less protective against smoking injury.

We found linkage to BHR and association with IgE and with the other phenotypes with decreasing P values, respectively.

The higher significance value for IgE level in the TDT has to be intended as a probability of observing by chance the results and not as the increased strength of disequilibrium association of this phenotype relative to the others. In fact, the number of transmitted and non-transmitted alleles is not very different for BHR and IgE. The association between the mutation and disease could be due to an involvement of ACT in the ethiopathogenesis or progression of allergic asthma or to linkage disequilibrium with a different susceptibility gene located close to the gene encoding ACT, which could be represented either by AT or by another locus.

In conclusion, linkage and association results from the Italian family data set study suggest that the gene encoding ACT or a closely located gene may be involved in susceptibility to allergic asthma in this group of patients.

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