

DNA Microarray Analysis of Chromosomal Susceptibility Regions to Identify Candidate Genes for Allergic Disease: A Pilot Study

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Objective—To examine whether DNA microarray analysis of chromosomal susceptibility regions for allergy can help to identify candidate genes.

Material and Methods—Nasal biopsies were obtained from 23 patients with allergic rhinitis and 12 healthy controls. RNA was extracted from the biopsies and pooled into three patient and three control pools. These were then analysed in duplicate with DNA microarrays containing 12 626 genes. Candidate genes were further examined in nasal biopsies (real-time polymerase chain reaction) and blood samples (single nucleotide polymorphisms) from other patients with allergic rhinitis and from controls.

Results—A total of 37 differentially expressed genes were identified according to criteria involving both the size and consistency of the gene expression levels. The chromosomal location of these genes was compared with the chromosomal susceptibility regions for allergic disease. Using a statistical method, five genes were identified in these regions, including serine protease inhibitor, Kazal type, 5 (SPINK5) and HLA-DRB2. The relevance of these genes was examined in other patients with allergic rhinitis and in controls; none of the genes were differentially expressed in nasal biopsies. Moreover, no association between allergic rhinitis and SPINK5 polymorphisms was found, at either the genotype or haplotype level.

Conclusions—DNA microarray analysis of chromosomal susceptibility regions did not lead to identification of candidate genes that could be validated in a new material. However, because gene polymorphisms may cause differential gene expression, further studies, including validation data, are needed to examine this approach. *Key words:* allergic rhinitis, expression profiling, linkage analysis, microarray.

INTRODUCTION

Complex diseases such as allergy depend on multiple interacting genes. Linkage scans have identified > 20 chromosomal susceptibility regions for allergic disease (1–3). Finding actual susceptibility genes is complicated both by the number of regions and by the fact that they may contain hundreds of genes (4). Recent studies (5, 6) indicate that polymorphisms in regulatory elements of the genome are common and an important cause of pathological changes in gene expression. Because linkage scans identify chromosomal regions with disease-related polymorphisms, differentially expressed genes that map to such regions may be candidate genes. DNA microarrays can be used to identify changes in gene expression on a genome-wide scale (7, 8). Thus, DNA microarray analysis of chromosomal susceptibility regions may help to find candidate genes. This approach was tried in a DNA microarray study of skin biopsies from patients with psoriasis and healthy controls (9) and led to the identification of new candidate genes. However, the relevance of these genes was not examined in a new material.

The objective of this study was to combine linkage and DNA microarray analysis to study human allergic disease (Fig. 1A). The chromosomal location of differentially expressed genes was compared with the most consistently described chromosomal susceptibility regions, i.e. 5q31–33, 6p21, 11q13–21, 12q21–24 and 13q12–14 (2, 3), and a statistical method was developed to identify susceptibility genes among differentially expressed genes. Moreover, in a second validation step, a new material was examined for both differential expression and polymorphisms of the candidate genes.

MATERIAL AND METHODS

Study design

A total of 23 patients with symptomatic birch pollen-induced allergic rhinitis and 12 healthy controls were enrolled prospectively. All the subjects were Swedish. The patients were seen after they developed symptoms during the birch pollen season in 2000. The controls were also seen during the birch pollen season. The median ages of the patients and controls were 42

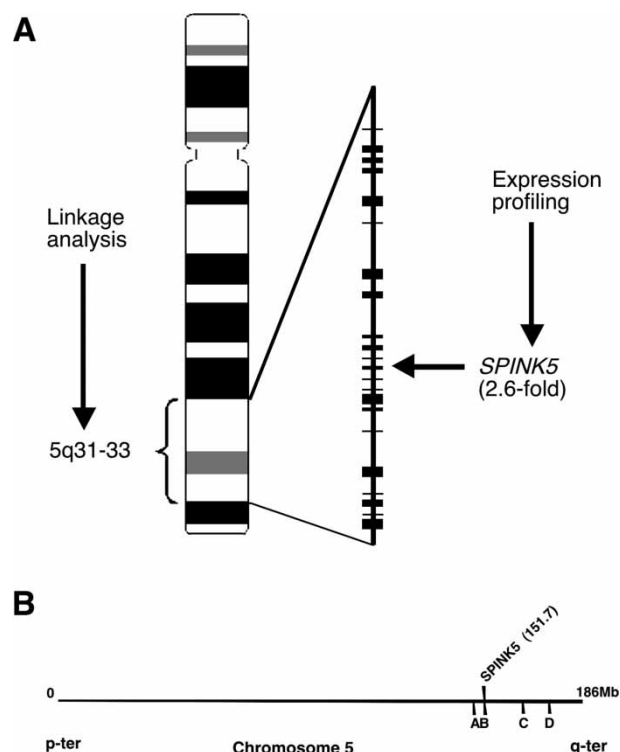


Fig. 1. (A) Combined linkage and DNA microarray analysis to identify susceptibility genes. Linkage studies have identified chromosomal susceptibility regions for allergic disease, e.g. 5q31–33. This region contains 106/6224 genes in the reduced dataset. Sequence variation in one or more of these genes may cause pathological changes in expression. As a result, differentially expressed genes that map to chromosomal susceptibility regions may be susceptibility genes. In this study, DNA microarray analysis revealed that the expression of *SPINK5* was 2.6-fold lower in patients with allergic rhinitis than in healthy controls. *SPINK5* is located within 5q31–33, and may therefore be a susceptibility gene. (B) Location of the *SPINK5* gene in 5q32 relative to the suggested asthma/allergy loci in chromosome region 5q31–33. The genome map for chromosome 5 on the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>) comprises 186 Mb. *SPINK5* is located in position 151.7 Mb from 5pter. Several published genome scans for asthma/allergy have suggested linkage to chromosome 5q regions, e.g. close to markers D5S436 (A; 148.0 Mb from pter), D5S1480 (B; 150.9 Mb), D5S820 (C; 165.2 Mb) and D5S1471 (D; 174.4 Mb). The span of the suggested regions is ≈ 25 cM in size in several of these scans, and the *SPINK5* gene thus maps well within the suggested asthma/allergy region on 5q31–33.

(range 18–69 years and 31 (24–66) years, respectively. Nasal biopsies from these patients were analysed using DNA microarrays. To validate the results from the DNA microarray analysis, nasal biopsies from a new group of six patients and six healthy controls were obtained during the pollen season in the following year. Moreover, blood samples were collected from 16 patients and 28 controls. All the patients were symptomatic and had a positive history of seasonal

allergic rhinitis for at least 2 years. Patients were excluded if they had a history of perennial rhinitis, had positive skin prick tests against perennial allergens, had been given local or systemic glucocorticoids or antihistamines before the study or were pregnant. The controls were all symptom-free, and had no history of allergic rhinitis or any other atopic disease. Patients and controls were examined using skin prick tests against a panel of allergens, including birch, timothy, mugwort, house dust mite (*Dermatophagoides pteronyssinus* and *D. farinae*), horses, dogs, cats and moulds (*Cladosporium* and *Alternaria*). All the patients had positive skin prick tests against birch, whereas the controls had negative skin prick tests against the entire panel of allergens. The study was approved by the Ethics Committee at the Medical Faculty, Lund University.

Preparation of cRNA, hybridization to DNA microarrays and data analysis

RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). The RNA was pooled into three patient and three control pools. Each pool was divided and analysed in duplicate as described previously (8). Briefly, RNA was transcribed into cDNA (Life Technologies, Rockville, MD) and biotin-labelled cRNA (Enzo, Farmingdale, NY) was resynthesized. Hybridization to DNA microarrays (Human Genome U95A array version 2; Affymetrix, Santa Clara, CA) and detection of hybridized target cRNA was performed according to the Affymetrix Gene Chip Expression Analysis manual. The quality of the cDNA synthesis and in vitro transcription was assessed by hybridization to Test2 arrays (Affymetrix). Scanned output files were visually inspected for hybridization artefacts and then analysed using Microarray Suite 5.0 software (Affymetrix). The overall performance of the DNA microarrays was assessed using different control experiments: the hybridization quality was supported by the four control bacterial and phage gene cRNAs being present on all DNA microarrays; the quality of in vitro transcription and cDNA synthesis was indicated by the 5' expression levels of actin and glyceraldehyde-3-phosphate dehydrogenase being $> 70\%$ of the 3' intensities. To permit the comparison of gene expression between patients and controls, the arrays were scaled to an average intensity of 500. RNA expression was quantitatively estimated by computing the average difference between a set of oligonucleotides that perfectly matched the gene and a set of mismatch control oligonucleotides. The average difference value will subsequently be referred to as the gene expression level and given in arbitrary units. In addition, a qualitative estimate of gene expression was given by "the absolute call". This

was obtained using an algorithm based on the signal intensity and quality of the average difference (Affymetrix). Using the absolute call, gene expression is classified as absent, marginal or present. Transcripts that were scored absent for all arrays and were without a known chromosomal location were excluded.

Quantitative reverse transcriptase polymerase chain reaction

Two-tube quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was utilized throughout the study, i.e. the RT and PCR steps were performed in different tubes. Reverse transcription of total RNA to cDNA was carried out using the OmniscriptTM RT kit (Qiagen GmbH, Germany) in a 20- μ l reaction volume using a Mastercycler personal PCR machine (Eppendorf AG, Germany) at 37°C for 1 h. Quantitative real-time PCR assays were performed using the ABI PRISM[®] 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Oligonucleotide primers for PCR assays were designed using Prime Express[®] 2.0 software (Applied Biosystems). Primer sequences are listed in Table I (online supplement) and were synthesized by DNA Technology A/S (Aarhus, Denmark). PCR was performed using the QuantiTect SYBR[®] Green RT-PCR kit (Qiagen) in a final volume of 50 μ l. PCR conditions were as follows: incubation at 95°C for 15 min, followed by 40 cycles at 94°C for 30 s and at 55°C for 60 s. Samples were assayed in triplicate. The data were analysed using the comparative cycle threshold (Ct) method and the real-time PCR products were visualized using agarose electrophoresis. A blank (no template) was included in all the experiments as a negative control.

Gene expression changes were assessed using the comparative Ct method. The relative amounts of mRNA for uteroglobin and mammoglobin B were determined by subtracting the Ct values for these genes from the Ct value for β -actin (housekeeping gene). Δ Ct values were generated in this way for each sample and, subsequently, the mean Δ Ct value for the control group was subtracted from the mean Δ Ct value for the treated group ($\Delta\Delta$ Ct). Squaring these data gives the relative gene expression in fold changes between the control and treatment groups (<http://docs.appliedbiosystems.com/pebi docs/04303859.pdf>).

Molecular studies of the single nucleotide polymorphisms in the SPINK5 gene

Association to allergic rhinitis and three polymorphisms in SPINK5 was analysed in DNA from 16 patients with allergic rhinitis and from 28 healthy controls. The polymorphisms were chosen based on the work of Walley et al. (10). Primers for DNA

sequencing were designed using Oligo[®] Primer Analysis Software version 6.0 (Molecular Biology Insights, Inc., Cascade, CO). For the single nucleotide polymorphisms (SNPs) 1103A/G (Genbank accession number AJ228139) and 1156 G/A in exon 13, the following primers were used: forward, 5'-TCCCTGACACCTTCCC-3'; reverse, 5'-TCCCTCCATGTTCCCA-3'. For the SNP 1258 G/A in exon 14 the following primers were used: forward, 5'-CACAGG-GTTAGGCACA-3'; reverse, 5'-AGGAATGCACGTGTTTC-3'. Following initial denaturation at 95°C for 30 s, amplifications were conducted for 30 cycles at 95°C for 30 s, 57–62°C for 30 s and 72°C for 1 min, with a final extension step at 72°C for 7 min. DNA sequencing was carried out with the forward primer as the sequencing primer using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequences were analyzed using an ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

Selection of chromosomal susceptibility regions

Several gene regions with a linkage to allergy have been presented in a number of genome scan studies and some of these regions have also been replicated by other groups. They include regions 5q31–33 (IL-4 cytokine cluster) (11, 12), 6p21 [the major histocompatibility complex (MHC)/human leukocyte antigen (HLA) region] (11–17), 11q13–21 (close to the high-affinity receptor for IgE) (12–14, 17), 12q21–24 (14, 15) and 13q12–14 (11, 13). Differentially expressed genes from the DNA microarrays mapping to these specific regions, as determined by the information given by the chip supplier (Affymetrix), were studied. These locations were also checked by Basic Local Alignment Search Tool searches of the respective cDNAs using the University of California at Santa Cruz Genome Bioinformatics website, June 2002 version (<http://genome.ucsc.edu>).

Statistical analysis

To test the hypothesis of independence between genes that are differentially expressed and genes that are located in susceptibility regions a score test is suggested. This method consists of both a criterion for identifying genes that are differentially expressed and a score test to see whether a significant number of the differentially expressed genes are located within linkage susceptibility regions. The criterion for differential expression involved both the size of the fold change and the consistency of the gene expression levels within the patient and control groups. Specifically, we required the fold change to be larger than two and that a *t*-test on logarithms of the signal values should

reveal a significant difference between the patient and control groups at the 5% level.

The chromosomal location of the differentially expressed genes was compared with the most consistently described chromosomal susceptibility regions, i.e. 5q31–33, 6p21, 11q13–21, 12q21–24 and 13q12–14. The score test was performed as follows. Let G_2 denote the number of genes in the susceptibility regions. For each differentially expressed gene the score equals the absolute value of the logarithm of the fold change and for other genes the score is zero. The test variable S is the sum of the score values for genes in the linkage susceptibility regions. The corresponding p -value is the probability of obtaining an S -value that is at least as large in random samples of G_2 genes from the set of all the genes in the reduced dataset adding up the scores for the genes that are sampled. A detailed description of the score test is given at the online supplement.

RESULTS

General characteristics of the gene expression data

Nasal biopsies from patients with symptomatic allergic rhinitis and healthy controls were analysed using duplicate DNA microarrays that measured the expression of 12 626 genes. The reproducibility of the gene expression data was supported by good agreement between the duplicate microarrays. The correlation coefficients, r , of the log expression levels for the control microarrays ranged from 0.92 to 0.94. The corresponding r -values for the patient microarrays were 0.93–0.96. Of the original 12 626 genes in the DNA microarray analysis, 6224 remained after excluding genes that were absent according to the absolute call criterion or had no known chromosomal location. These genes will be referred to as G_1 .

DNA microarray analysis of chromosomal susceptibility regions

A total of 37 differentially expressed genes were identified according to a criterion involving both the size and consistency of the gene expression levels (Table II, online supplement). The chromosomal location of these genes was compared with the chromosomal susceptibility regions for allergic disease, i.e. 5q31–33, 6p21, 11q13–21, 12q21–24 and 13q12–14. Five of the 37 genes were located within these regions (Table III, online supplement). Of the 6224 G_1 genes in the reduced dataset, 536 G_2 genes were located in the susceptibility regions. A score test was applied to test the hypothesis of independence between genes that were differentially expressed and genes that were located in susceptibility regions. For the S -value corresponding to the observations in Table

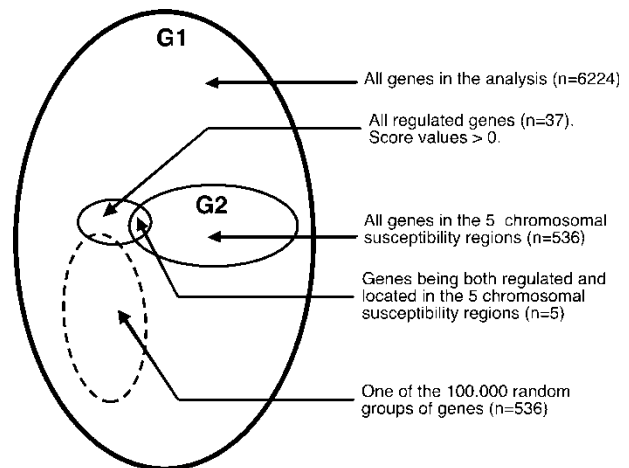


Fig. 2. Graphical description of the statistical model used to test the hypothesis of independence between genes that are differentially expressed and that are located in susceptibility regions. Random samples are drawn and score values are added for the genes in the sample. The p -value is the relative frequency of samples for which the score sum is at least as large as the score sum for genes in the linkage susceptibility regions.

II this gave a p -value of 3.7% (computed by simulation with 1 200 000 runs) and the independence hypothesis was rejected (Fig. 2). The test thus suggests that at least one of the five genes found in linkage regions is associated with the disease. The functions of the five genes were examined using searches of the ExPASy Molecular Biology Server (<http://www.expasy.ch>) and the Online Mendelian Inheritance in Man (OMIM; <http://www.ncbi.nlm.nih.gov>) databases. Two of the five identified genes—HLA-DRB2 and serine protease inhibitor, Kazal type, 5 (SPINK5)—have known functions relevant to allergy and both had lower expression in patients than controls. HLA-DRB2 is involved in antigen presentation to CD4 surface protein on T cells. SPINK5 encodes lymphoepithelial Kazal-type-related inhibitor (LEKTI), a serine protease inhibitor that protects the airways against inflammatory damage.

Quantitative RT-PCR analysis of SPINK5 and HLA-DRB2

To compare the expression in nasal biopsies from a new set of patients and healthy controls ($n = 6$ in each group), the levels of SPINK5 and HLA-DRB2 were related to the expression of the reference 18S. $\Delta\Delta C_t$ analysis of the data demonstrated no difference in expression of the SPINK5 gene in patients (-0.24 ± 1.32 -fold change; $p = 0.75$). Similarly, for the expression of the HLA-DRB2 gene, no difference was obtained in patients with rhinitis (-0.13 ± 1.45 -fold change; $p = 0.80$).

Test for association between SPINK5 SNPs and allergic rhinitis

To test for association between allergic rhinitis and genetic polymorphisms in the SPINK5 gene, DNA from 16 patients with allergic rhinitis and 28 healthy controls was analysed using 3 SNPs in the SPINK5 gene. Using DNA sequencing, genotypes could be determined and haplotypes deduced. We could show that no association was present between allergic rhinitis and the three polymorphisms studied in SPINK5, using either the genotype data (Table IV) or using haplotypes (Table V).

DISCUSSION

Allergic diseases depend on interactions between multiple susceptibility genes. It is not known how many of the 30 000 human genes are involved. Genome-wide linkage scans have led to the identification of chromosomal regions that may contain susceptibility genes (1–3). Finding these genes is complicated by the number and width of the regions, each of which may contain hundreds of genes (4). Recent data suggest that a complementary approach may help to identify candidate genes. Polymorphisms involved in the development of complex disorders are often located in regulatory regions of the genome and therefore affect the transcription of the candidate gene. It has been estimated that a large fraction of all genes (5–40%) have variations in regulatory regions that affect gene expression 1.5-fold or greater (5, 6). This suggests that DNA microarray analysis of chromosomal susceptibility regions could help to identify candidate genes.

This approach was tried in this study of nasal biopsies from patients with allergic rhinitis and healthy controls. A statistical model was developed to find differentially expressed genes within chromosomal susceptibility regions. The relevance of the identified genes was examined by analysing their expression in a new material. Moreover, SNP analysis of one of the genes was performed. Two of the five identified genes have known functions relevant to allergy; one of these is SPINK5, encoding LEKTI, a serine proteinase inhibitor that is expressed in mucosal epithelium and in the thymus (18). To our knowledge, local expression of SPINK5 has not been described in the mucosa of patients with allergic disease. In this material, SPINK5 expression was lower in patients than controls. The relevance of SPINK5 to allergic disease is supported by many observations. A reduction in the expression of SPINK5 may contribute to allergic inflammation by reducing airway protection and changing the maturation of lymphocytes (18). Mutations in SPINK5 cause Netherton's syndrome, a

recessive disorder involving dermatitis, asthma and allergic rhinitis (19). SPINK5 polymorphisms are associated with allergic disease (10), and the SPINK5 gene is located within the asthma region on chromosome 5q31–33, as proposed by several genome scans (Fig. 1B).

The expression of HLA-DRB2 was also reduced in the patients. The class II regions of the MHC present antigens to the CD4 surface protein on T cells. Reduced HLA-DR expression has been reported in patients with asthma (20). Interestingly, solubilized complexes of HLA-DR2 and antigen have been tried therapeutically in autoimmune diseases (21). Taken together, these findings support the fact that SPINK5 and HLA-DRB2 could be candidate genes for allergic disease.

Although a new statistical method was developed to identify the candidate genes, the small size of the material indicates that the results should be interpreted with caution. While a larger material would have been better, it should be noted that there are considerable practical and ethical concerns with obtaining nasal biopsies, mainly the risk of significant bleeding. This risk is proportional to the size of the biopsy. To obtain sufficient RNA for the DNA microarray examination, the samples had to be pooled. This may result in the gene expression levels being skewed by individuals with extreme values. To reduce this problem we now believe that it would have been better to analyse twice as many pools using single DNA microarrays, rather than having used duplicate DNA microarrays. This is supported by good correlations between the duplicate analyses as well as independent studies confirming the validity of the DNA microarrays (22). However, the small size of the material led us to undertake a second (validation) step, in which the relevance of the candidate genes was examined in a new material. In this second step, neither SPINK5 nor HLA-DRB2 were found to be differentially expressed. Moreover, three SPINK5 SNPs previously described in patients with allergic disease were examined. No association between allergic rhinitis and the SPINK5 polymorphisms was seen, at either the genotype or haplotype level. This, however, does not rule out a role for SPINK5 polymorphisms in allergic rhinitis, as only three polymorphisms were studied.

There could be other methodological and biological explanations for the difficulties in validating the candidate genes in new materials. The most obvious is that the two genes were differentially expressed secondary to environmental influence. Indeed, the materials were collected during different pollen seasons. Moreover, changes in gene expression could be secondary to influx of inflammatory cells during the

pollen season, rather than representing primary differences between patients and controls. Further studies of patients out of season are therefore needed. Another concern is the relevance of the chromosomal susceptibility regions to allergic disease. Although the most consistently described regions were examined in this study, > 20 different regions have been described. This could depend on both ethnic and individual heterogeneity but suggests that many different combinations of genes could lead to the same phenotype. Thus, candidate genes in one study may not be relevant in a new material. Most linkage studies have been performed on asthmatic patients, and this study focused on allergic rhinitis. While asthma and allergic rhinitis are pathophysiologically and epidemiologically linked (22, 23) it is possible that combined DNA microarray and linkage analysis of samples from asthmatic patients would be more likely to reproducibly identify new candidate genes.

The problems discussed above reflect the scale and complexity of identifying candidate genes in allergic disease. It is not known how many of the 30 000 human genes are involved. Moreover, different genes could contribute to the same disease in different individuals. Thus, identification of candidate genes using either linkage or DNA microarray analysis is a formidable task.

In summary, DNA microarray analysis of chromosomal susceptibility regions may help to identify candidate genes for allergic diseases. However, confirmation of the results in a new material is required.

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REFERENCES

1. Pawliczak R, Shelhamer JH. Application of functional genomics in allergy and clinical immunology. *Allergy* 2003; 58: 973–80.
2. Cookson WO, Moffatt MF. Genetics of asthma and allergic disease. *Hum Mol Genet* 2000; 9: 2359–64.
3. Hakonarson H, Wjst M. Current concepts on the genetics of asthma. *Curr Opin Pediatr* 2001; 13: 267–77.
4. Terwilliger JD, Shannon WD, Lathrop GM, Nolan JP, Goldin LR, Chase GA, et al. True and false positive peaks in genome wide scans: applications of length-biased sampling to linkage mapping. *Am J Hum Genet* 1997; 6: 430–8.
5. Cowles CR, Joel NH, Altshuler D, Lander ES. Detection of regulatory variation in mouse genes. *Nat Genet* 2002; 32: 432–7.
6. Yan H, Yuan W, Velculescu VE, Vogelstein B, Kinzler KW. Allelic variation in human gene expression. *Science* 2002; 297: 1143.
7. Wohlfahrt JG, Kunzmann S, Menz G, Kneist W, Akdis CA, Blaser K, et al. T cell phenotype in allergic asthma and atopic dermatitis. *Int Arch Allergy Immunol* 2003; 131: 272–82.
8. Benson M, Cardell LO, Carlsson B, Jernås M, Reinholdt J, Svensson PA, et al. DNA microarrays to study gene expression in allergic airways. *Clin Exp Allergy* 2002; 30: 1–8.
9. Bowcock AM, Shannon W, Du F, Duncan J, Cao K, Aftergut K, et al. Insights into psoriasis and other inflammatory diseases from large-scale gene expression studies. *Hum Mol Genet* 2000; 10: 1793–805.
10. Walley AJ, Chavanas S, Moffatt MF, Esnouf RM, Ubhi B, Lawrence R, et al. Gene polymorphism in Netherton and common atopic disease. *Nat Genet* 2001; 29: 175–8.
11. Koppelman GH, Stine OC, Xu J, Howard TD, Zheng SL, Kauffman HF, et al. Genome-wide search for atopy susceptibility genes in Dutch families with asthma. *J Allergy Clin Immunol* 2002; 109: 498–506.
12. Haagerup A, Bjerke T, Schiotz PO, Binderup HG, Dahl R, Kruse TA. Asthma and atopy—a total genome scan for susceptibility genes. *Allergy* 2002; 57: 680–6.
13. Daniels SE, Bhattacharya S, James A, Leaves NI, Young A, Hill MR, et al. A genome-wide search for quantitative trait loci underlying asthma. *Nature* 1996; 383: 247–50.
14. Xu J, Meyers DA, Ober C, Blumenthal MN, Mellen B, Barnes KC, et al. The Collaborative Study on the Genetics of Asthma. Genome wide screen and identification of gene-gene interactions for asthma-susceptibility regions in three U.S. populations: collaborative study on the genetics of asthma. *Am J Hum Genet* 2001; 68: 1437–46.
15. Wjst M, Fischer G, Immervoll T, Jung M, Saar K, Rueschendorf F, et al. A genome-wide search for linkage to asthma. German Asthma Genetics Group. *Genomics* 1999; 58: 1–8.
16. Hizawa N, Freidhoff LR, Ehrlich E, Chiu YF, Duffy DL, Schou C, et al. Genetic regulation of Dermatophagoides pteronyssinus-specific IgE responsiveness: a genome-wide multipoint linkage analysis in families recruited through 2 asthmatic sibs. Collaborative Study on the Genetics of Asthma (CSGA). *J Allergy Clin Immunol* 1998; 102: 436–42.
17. Dizier MH, Besse-Schmittler C, Guilleud-Bataille M, Annesi-Maesano I, Boussaha M, Bousquet J, et al. Genome screen for asthma and related phenotypes in the French EGEA study. *Am J Respir Crit Care Med* 2000; 162: 812–8.
18. Magert HJ, Standker L, Kreutzmann P, Zucht HD, Reinecke M, Sommerhoff CP, et al. LEKTI, a novel 15-domain type of human serine proteinase inhibitor. *J Biol Chem* 1999; 274: 21499–502.
19. Chavanas S, Bodemer C, Rochat A, Hamel-Teillac D, Ali M, Irvine AD. Mutations in SPINK5, encoding a

- serine protease inhibitor, cause Netherton syndrome. *Nature Genet* 2000; 25: 141–2.
20. Kraft M, Striz I, Georges G, Umino T, Takigawa K, Rennard S, et al. Expression of epithelial markers in nocturnal asthma. *J Allergy Clin Immunol* 1998; 102: 376–81.
21. Goodkin DE, Shulman M, Winkelhake J, Waubant E, Andersson P, Stewart T, et al. A phase I trial of solubilized DR2:MBP84-102 (AG284) in multiple sclerosis. *Neurology* 2000; 54: 1414–20.
22. Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ, Sealfon SC. Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. *Nucleic Acids Res* 2002; 30: e48.
23. Simons FER. Allergic rhinobronchitis: the asthma-allergic rhinitis link. *J Allergy Clin Immunol* 1999; 104: 534–40.
24. Djukanovic R, Wilson SJ, Howarth PH. Pathology of rhinitis and bronchial asthma. *Clin Exp Allergy* 1996; 26(Suppl 3): 44–51.

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