

Gene polymorphism in Netherton and common atopic disease

Andrew J. Walley¹, Stéphane Chavanas¹, Miriam F. Moffatt¹, Robert M. Esnouf¹, Baljinder Ubhi¹, Robert Lawrence¹, Kenny Wong¹, Gonçalo R Abecasis¹, E. Yvonne Jones¹, John I. Harper², Alain Hovnanian¹ & William O.C.M. Cookson¹

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Atopic dermatitis (AD) and asthma are characterized by IgE-mediated atopic (allergic) responses to common proteins (allergens), many of which are proteinases. Loci influencing atopy have been localized to a number of chromosomal regions¹, including the chromosome 5q31 cytokine cluster²⁻⁴. Netherton disease is a rare recessive skin disorder in which atopy is a universal accompaniment⁵. The gene underlying Netherton disease (*SPINK5*)⁶ encodes a 15-domain serine proteinase inhibitor (LEKTI) which is expressed in epithelial and mucosal surfaces and in the thymus^{6,7}. We have identified six coding polymorphisms in *SPINK5* (Table 1) and found that a Glu420→Lys variant shows significant association with atopy and AD in two independent panels of families. Our results implicate a previously unrecognized pathway for the development of common allergic illnesses.

We examine association to *SPINK5* in a primary panel of families recruited through children with AD (the GOS panel). The mean age of the children was 6.9 years (SD ± 3.9). Of the 338 children studied, 172 were male and 166 female; 254 children had AD, 153 had asthma and 139 had both; and 40% of the children had IgE levels above the normal limit of 87.3 kU/l (ref. 8). The log_e normalized total serum IgE in the children is bi-modally distributed, with a first peak at approximately 50 kU/l and the second at 3,000 kU/l.

We used transmission disequilibrium tests to study associations with coding polymorphisms with a minor allele frequency of greater than 10% (Asn368→Ser, Asp386→Asn and Glu420→Lys). Inspection of the raw transmission data indicates the possible presence of parent-of-origin effects (Table 2a). We therefore tested the significance of association using a previously described method⁹ which computes a robust parent-of-origin likelihood-ratio statistic for diseases for which there could be maternal effects on risk.

Our test of significance of difference between maternal and paternal allele sharing confirms the presence of parent-of-origin effects for Asn368 and Lys420 alleles in children with atopy ($\chi^2=11.5$ (1 df), $P=0.008$ and $\chi^2=12.9$ (1 df), $P=0.002$ respectively). Maternally inherited Asn368 and Lys420 alleles are significantly associated with increased risk of atopy (Table 2a) when compared with paternally transmitted alleles. The relative risk associated with maternal inheritance of these alleles is approximately four. We observed similar associations to AD and the total serum IgE concentration (Table 2a). There is a weak association between Lys420 and asthma; paternal and maternal alleles are equally informative in the tests of association.

We also carried out an empirical test of significance to allow for possible biases due to the testing of multiple affected offspring and phenotypes per family. In this test, the transmission of

parental chromosomes is randomly permuted 1,000 times and the most significant association to any phenotype in each replicate is counted. The overall probability of finding an association significant as observed with the phenotypes tested is 0.048.

To further establish that the results were truly significant, we tested a secondary panel of families (UK1) for Asn368→Ser and Glu420→Lys. UK1 contained 215 offspring, of which 131 were atopic, 70 had questionnaire-diagnosed AD and 120 were

Table 1 • Single-nucleotide polymorphisms in *SPINK5*

Exon	SNP ^a	Amino-acid change	Frequency ^b
2	56–125 A→G		0.38 A
	81+54 G→A		0.34 G
3	82–31: A→G		0.38 A
5	316: G→A	Asp106→Asn	0.03 A
7	475–86: G→C		0.44 G
12	1011–12: C→T		0.50 T
13	1093–26: C→T		0.47 C
	1093–10: A→G		0.47 A
	1103: A→G	Asn368→Ser	0.50 A
	1156: G→A	Asp386→Asn	0.13 A
	1188: T→C	His396	0.47 T
14	1258: G→A	Glu420→Lys	0.48 A
	1221–50: G→A		0.50 A
15	1389: A→G	Gly463	0.50 G
16	1431–49 A ₁₀ →A ₁₁		0.09 A ₁₁
	1431–51 A→G		0.03 G
17	1557: C→A	Gly519	0.22 A
	1607+47: C→T		0.09 T
18	1659: C→T	Val553	0.50 T
20	1821–47: T→G		0.09 G
21	1888–54: G→A		0.38 G
24	2241–26: T→C		0.44 T
	2313+31: C→G		0.34 C
	2313+48: G→A		–
25	2358: C→T	Leu786	0.44 C
	2412: C→T	Gly804	0.44 C
26	2475: G→T	Glu825→Asp	0.08 T
28	2667–13 A→T		0.31 A
29	2740–59: G→A		0.50 A
30	2915: A→G	His972→Arg	0.04 G
31	2965–46: T→C		0.44 T
	3009 C→T	Gly1003	0.44 T

^aSNPs are numbered according to their position relative to the published cDNA (GenBank accession number AJ228139). Where they are intronic, they are numbered + or – the nucleotide at the nearest exon-intron boundary. Position 1 is the A of the initiation codon. ^bFrequency of minor allele (by convention, allele 2).

¹Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Headington, Oxford OX3 7BN, UK. ²Department of Paediatric Dermatology, Great Ormond Street Hospital for Children, London, UK. Correspondence should be addressed to W.O.C.M.C. (e-mail: wocc@well.ox.ac.uk).



Table 2 • Weinberg tests of association

a 148 nuclear families with atopic dermatitis: parent-of-origin effects included in model

Marker	Phenotype	Disease allele	Transmissions (T:NT) ^a		P	Rm ^b
			Maternal	Paternal		
Glu420→Lys	Atopy	Lys420	46:28	32:45	0.005	4.04
Glu420→Lys	AD	Lys420	44:31	33:47	0.009	3.92
Glu420→Lys	IgE**	Lys420	46:28	32:45	0.005	4.25
Glu420→Lys	Asthma	Lys420	29:16	23:25	0.070	3.1
Asn368→Ser	Atopy	Asn368	49:27	36:42	0.023	3.47
Asn368→Ser	AD	Asn368	47:28	36:43	0.024	3.67
Asn368→Ser	IgE ^c	Asn368	49:28	36:42	0.030	3.55

b 66 nuclear and 7 extended families in replication panel: parent-of-origin effects included in model

Marker	Phenotype	Disease allele	Transmissions (T:NT) ^a		P	Rm ^b
			Maternal	Paternal		
Glu420→Lys	Atopy	Lys420	15:8	18:6	0.008	2.63
Glu420→Lys	AD	Lys420	10:1	15:3	0.002	3.32
Glu420→Lys	IgE ^c	Lys420	14:7	18:7	0.012	2.35
Glu420→Lys	Asthma	Lys420	11:4	17:10	0.040	1.43

^aUnambiguous transmissions only: note the Weinberg test also uses information from doubly heterozygous parents. ^bRm=Relative risk of transmission of maternal alleles compared to paternal alleles. ^cDichotomized about a value of 87.3 kU/l⁸.

asthmatic. We saw significant associations of maternally derived alleles between atopy, AD, asthma and the total serum IgE and Lys420 (Table 2b), but we did not observe associations with Asn368. Permutation tests show that these associations are also unlikely to result from biases due to multiple affected offspring within families (permutation *P* value=0.004 for AD and 0.012 for all phenotypes together). By contrast with the first panel of families, paternally derived alleles tend to be positively associated with disease, although less so than maternal alleles (Table 2b).

represent a different disease than AD alone⁴; differential association of subgroups of disease to *SPINK5* should therefore be explored in larger datasets. In general, the associations in our panels seem strongest to AD and to atopy, with weaker correlations with asthma. Notably, a strongly positive TDT test with a 'loose' definition of asthma has been previously observed in Hutterite families at the marker *D5S1480* (position 147.5 cM)¹⁴, which may be in linkage disequilibrium with *SPINK5* in that founder population.

Although parent-of-origin effects have not been reported with markers from the proximal cytokine cluster, they have been

SPINK5 is at the distal end of a cytokine cluster that extends approximately from *D5S490* (134 cM from the top of the chromosome) to *CSF1R* (position 153 cM). The region is thought to contain more than one locus influencing levels of IgE^{3,10}, and observations of association have been made to genes that are quite proximal to *SPINK5*, including *IL4* (position 136 cM)¹¹, *IL13* (position 136 cM)¹² and *CD14* (position 141 cM)¹³.

Our study examines several correlated phenotypes. It is not clear whether *SPINK5* polymorphism is primarily associated with AD, asthma, or the general atopic state. It is also possible that AD and asthma together



Fig. 1 Amino-acid sequence of the LEKTI protein, modified from previous description⁷. The protein has 15 domains. Domains 2 and 15 are shown in orange and contain a typical Kazal-Type (KT) arrangement of six cysteine residues which are shown in dark blue. The other 13 domains form a distinct subset of KT sequences (shown in blue) with four cysteine residues and well-conserved proteinase inhibitory sequences between the second and third cysteines. Numbered exon boundaries are shown below the sequence. The central parts of each domain are encoded by single odd-numbered exons. Linking regions are encoded by the even-numbered exons. An amino-terminal secretory sequence is shown in green. Domains 1 and 6 have been identified free in plasma⁷, and are shown in red. Coding variants are shown in cerise below the wildtype sequence.

observed at other loci influencing allergic disease^{15–20} and in other disorders^{21,22}. The strength of parent-of-origin effects in other disorders varies among family collections²², as seen in our panels; the risks we observed may be different in unselected families from the general population. These findings are not explicable through simple genomic imprinting, as Netherton disease is a mendelian recessive disorder. Mono-allelic expression of immune molecules is well recognized, however, including IL-4 from the same genomic region as the Netherton disease locus²³. It is therefore possible that differential expression of maternal and paternal alleles of *SPINK5* may be tissue-specific, for example in the thymus, and may take place at particular moments during immune development. The discovery of genes such as *SPINK5* and *MS4A2* (encoding Fc ϵ RI- β), which show parent-of-origin effects, will allow elucidation of the structural mechanisms for epigenetic influences on atopy.

LEKTI is encoded by 33 exons⁶ and is comprised of 15 Kazal-type (KT) proteinase inhibitor domains totalling 1,064 amino acid residues⁷ (Fig. 1). The linker regions of LEKTI contain many charged residues, which are likely to be solvent exposed in the full-length protein. The more hydrophobic KT domains may thus cluster within the intact molecule, shielding their proteinase inhibitory loops. Proteinases typically cut after basic residues (arginine (R) and lysine (K)), which are common in the LEKTI linker sequences (Fig. 1). Proteolysis of full-length LEKTI by extraneous proteinase may release the KT domains, triggering a strong inhibitory response. In this context, it may be relevant that Glu420→Lys introduces an additional basic residue into the linker sequence between LEKTI domains 6 and 7.

There are a number of possible mechanisms for LEKTI to influence allergic disease. Proteinase-activated receptors are found in keratinocytes and can serve as targets for mast cell proteinases²⁴. Proteinases are involved in T and B cell maturation; the expression within the thymus⁷ of the gene underlying Netherton disease may indicate a role for LEKTI in these processes, or in antigen handling within other thymic cells. In addition, many allergens are also serine proteinases²⁵, and this proteinase activity may encourage their allergenicity in the presence of variable proteinase inhibition.

Methods

Subjects. We examined two panels of families. The primary panel (GOS panel) of 148 families containing 679 individuals was recruited from the dermatology clinics at the Great Ormond Street Hospital for Children, through a child or children with active AD. A physician examined each family for evidence of atopic dermatitis. We used a previously described scoring system²⁶ to assess the severity of AD. A physician-administered questionnaire, which included the diagnostic criteria for atopic dermatitis defined by the UK Working Party^{27,28} and a set of questions based on the American Thoracic Society's questionnaire for asthma and allergic rhinitis²⁹, was completed for each individual. We carried out skin prick tests for house dust mite (*Dermaphagoides pteromyssinus*) and timothy grass (*Phleum pratense*)²⁰. We measured total serum IgE concentration and the specific IgE to the same allergens by fluorescent enzyme immuno-assay (Pharmacia CAP system). We defined atopy as one of the following: (i) the presence of a positive skin prick test response 3mm greater than the negative control (ii) a positive specific IgE (iii) raised total serum IgE or (iv) any combination of these features (as previously described²⁹). We defined physician-diagnosed asthma as a positive response to the questions "Has your child ever had an attack of asthma?" and "Has your doctor ever told you that you have asthma?"

We tested positive associations for replication in an additional panel of 66 nuclear and 7 extended families recruited from clinics in the United Kingdom (UK1 Panel), which had been previously used to map atopy on chromosome 11q13 (refs. 18,30). We carried out phenotyping as for the primary sample of families, except that the diagnosis of AD was based on a positive response to the question "Have you ever had an itchy rash or eczema in the creases of your skin?"

Association analyses. We first established the presence of a parent-of-origin effect by a modified Weinberg likelihood test⁹, in which a model including ordered mating types and association including dominance is compared with a model that allows heterozygotes to be different depending on which parent is being tested. We then sought association of categorical phenotypes to the SNPs using the robust parent-of-origin likelihood-ratio test of Weinberg⁹. We dichotomized total serum IgE about a value of 87.3 kU/l, which defines the normal limit in the general population⁸, and examined it using the same likelihood-ratio test. To control for testing of multiple affected offspring and phenotypes per family, we randomly permuted transmitted and non-transmitted parental alleles for each family and counted the most significant association to any phenotype in each of 1000 replicates. The proportion of replicates exceeding the most significant of the original findings provided an empirical *P* value. We calculated permutation *P* values with a single tail for the replication set.

Identification of polymorphism. We sequenced *SPINK5* in 18 unrelated individuals with atopic dermatitis. Individual exons were sequenced by standard Big Dye protocols in an ABI 377. PCR primer sequences are available (<http://www.well.ox.ac.uk/asthma/public/index.html>). We exported electropherograms for analysis using PHRED and PHRAP to determine sequence quality and the presence of SNPs.

SNP typing by enzyme digestion of PCR products. We typed the Glu420→Lys polymorphism by *Hph*I digestion of a 304-bp exon 14 PCR product, using the following primers (forward and reverse): 5'-TGCAATTGTGAGGATTCACAG-3', and 5'-CCTGAACATGATCTGTGGATC-3'. We typed the Glu825→Asp polymorphism by *Fok*I digestion of a 252-bp exon 26 PCR product, using the following primers: 5'-TGACTGTGAGTCTTAAAGTAC-3', and 5'-GGGACAGAGTCAGCATTTAC-3'.

SNP typing by oligonucleotide ligation assays. The polymorphisms Asn368→Ser and Asp386→Asn were typed by the oligonucleotide ligation assay (OLA)³¹ using a PCR product of a region spanning exons 13 and 14 of the gene. Primer details are available (See Web Note A).

Note: Supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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