Chapter 9 | Identification and association of polymorphisms in the Interleukin 13 gene with asthma and atopy in a Dutch population

(Am J Respir Mol Cell Biol, accepted for publication)

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ABSTRACT

Asthma and atopy are related conditions that may share similar genetic susceptibility. Linkage studies have identified a region on chromosome 5q that contains biologic candidates for both asthma and atopy phenotypes, inclu ding several proinflammatory cytokines. Interleukin 13 (IL13), one of the candidate genes in the region, is directly involved in the regulation of IgE and has been associated with both asthma and atopy. We sought to identify new polymorphisms in the IL13 gene, and evaluated the involvement of a subset of these variants in asthma and atopy in a case-control study using probands and spouses from a Dutch asthma family study. IL13 was sequenced in 20 probands and 20 unaffected spouses, and ten polymorphisms were identified, 4 novel and 6 previously reported. Three SNPs were detected in the 5'-promoter region, 2 in intron 1, and 5 in exon 4. Only one of the exon 4 SNPs resulted in an amino acid change (Arg130Gln). We analyzed three single nucleotide polymorphisms (SNPs) in IL13 in an extended group of 184 probands and their spouses: one in the promoter region (-1111), the Arg130Gln (nucleotide position 4257), and a 3' UTR SNP (nucleotide position 4738). The most significant associations were observed to asthma (p=0.005), bronchial hyperresponsiveness (p=0.003), and skin-test responsiveness (p=0.03) with the –1111 promoter. These results provide evidence that variation in the IL13 gene is involved in the pathogenesis of asthma and atopy. Further investigation is required to determine which specific alleles or combination of alleles contribute to these phenotypes, and the possible downstream effects of the resulting change in IL13 levels or activity.

INTRODUCTION

Asthma is a common respiratory disease characterized by intermittent airways obstruction and respiratory symptoms that are caused by acute and chronic bronchial inflammation. Bronchial hyperresponsiveness (BHR) and total serum IgE levels are closely associated with the asthma phenotype and have a strong genetic component.[1-6] It has been well documented that the presence of atopy and BHR may precede the development of clinical asthma.[1-2] The development of asthma appears to be determined by the interaction between host susceptibility (genetics) and a variety of environmental exposures.

Numerous genetic studies have mapped an asthma and/or atopy susceptibi lity gene(s) to a region on chromosome 5q31-q33 in several populations (Dutch[5,7], Amish [8], American Caucasian[9], Hutterite[10], and British[11,12]) This region contains a cluster of proinflammatory cytokines important in immune regulation. Two members of this cluster, interleukin (IL) 4 and IL13, have been both genetically and functionally implicated in the pathogenesis of asthma and atopy.[13-17] These cytokines are produced by Th2 cells and are capable of inducing isotype class-switching of B-cells to produce IgE.[18] They also share a receptor component, IL4Rα, which has been shown to be an important factor in the development or expression of atopy and asthma.[19-22] Furthermore, both IL4 and IL13 mRNA and protein have been localized to the airways in allergic asthma.[23,24]

The IL13 receptor consists of one IL4Rα subunit and either a low-affinity IL13Rα1[25] or a high-affinity IL13Rα2 subunit.[26] The complete receptor for IL4 is composed of one IL4Rα subunit and an IL4Rγ subunit. Therefore, it is possible that different polymorphisms in these receptors, as well as in the IL4 and IL13 cytokines, contribute to the complex regulation of atopy or asthma phenotypes. Association studies with polymorphisms in IL13 have been performed using various atopy and asthma phenotypes in several populations. A promoter polymorphism was identified at position –1111 (referred to as position –1055 in their report) adjacent to the nuclear factor of activated T cells (NFAT) site and reported to be associated with allergic asthma in a Dutch population.[14] In addition, an Arg130Gln polymorphism in exon 4 has been shown to be associated with high total serum IgE levels [15,16], atopic dermatitis[15], and asthma[17] in German[15,16], American[16], British[17] and Japanese [17] populations. In an effort to further understand the contribution of IL13 to asthma and atopy phenotypes, we have sequenced the IL13 gene in probands with asthma and their unaffected spouses to identify new sequence variants. We also performed case-control association studies with three of these polymorphisms in this Dutch population, in which we have previously obtained evidence for linkage on chromosome 5q31 to bronchial responsiveness and total serum IgE levels.[5,6]

MATERIALS AND METHODS

Population

This genetically homogeneous population has been described in detail previously[5,7,27] Probands (local Caucasian Dutch patients with asthma) were originally studied between 1962-1975 at Beatrixoord Hospital, Haren, the Netherlands. At that time patients were diagnosed with asthma by the presence of characteristic symptoms, airways hyperresponsiveness, and reversibility of airway obstruction. Between 1990 and 1999, probands with asthma were restudied, together with their spouses (all Caucasians), children and available grandchildren. Briefly, all individuals underwent baseline spirometry and reversibility to 800 mg albuterol; bronchial responsiveness testing to histamine was performed using a 30 second inhalation protocol.[27,28] A subject was considered to display BHR if the provocative concentration of histamine producing a 20% fall in FEV_1 (PC_{20}) was < 32 mg/ml histamine. For atopy, subjects had intracutaneous skin testing with 16 common aeroallergens, which was considered positive if the maximum wheal diameter was > 5mm. In the first 92 families, total serum IgE levels were measured by solid-phase immunoassay (Pharmacia Diagnostics, Uppsala, Sweden). Duplicate measurements were made and the mean for each subject was used. If the duplicate samples differed by more than five percent the test was repeated. In the second set of 108 families, total IgE levels were measured by enzyme linked fluorescence assay (Mini Vidas, Biomerieux, Inc.). Although the entire families were ascertained for genetic linkage studies, the probands and spouses represent an appropriate cohort for case-control association studies, especially since they are of comparable ages and have experienced similar environmental exposures, which accounts for some of the known age related differences in the frequency of BHR, serum IgE levels, and skin-test responsiveness. A total of 184 probands and their spouses were used for this IL13 association study. This study was approved by the Medical Ethics Committee at the University of Groningen. All subjects provided written informed consent. DNA was isolated from lymphocytes using standard procedures.

DNA resequencing of IL13

Resequencing of the IL13 gene in 20 affected (probands) and 20 unaffected individuals (spouses) was performed by cycle sequencing of overlapping PCR-amplified DNA fragments covering the 5'-flanking region, exons 1 to 4 and intron 1. We decided on this sequencing strategy to identify SNPs that are most likely to alter the regulation or functional activity of IL13. Primers and fragment sizes are shown in Table 1 (PCR primer pairs were designed using Primer Express™ version 1.0; Perkin-Elmer Applied Biosystems). PCR amplifications were carried out in 10 ml volumes containing 1X GeneAmp® PCR buffer (Perkin Elmer Applied Biosystems), 20 ng of genomic DNA, 30 ng of each forward and reverse primer, 400 mM of each dNTP (Amersham-Pharmacia Biotech), 1.5 to 3 mM $MgCl_2$ (primer-depen-

dent) and 0.5 units AmpliTaq Gold™ (Perkin Elmer Applied Biosystems). Before sequencing, amplification products were incubated with shrimp alkaline phosphatase (0.5 units; Amersham Pharmacia Biotech) and exonuclease I (5 units; Amersham Pharmacia Biotech) at 37°C for 30 min, followed by heat inactivation at 80°C for 15 min. Amplification products were double-strand sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems). After cycle sequencing, unincorporated BigDye terminators were removed using Sephadex G-50 (Sigma) in Millipore multiscreen HV plates. Sequence reactions were analyzed using an ABI PRISM 377 sequencer (Perkin Elmer Applied Biosystems) and the resultant chromatograms were aligned and viewed using Phred/Phrap [29] and Consed [30] software. The GenBank accession numbers for the reference genomic and mRNA sequences used for the IL13 gene are reported in the footnote to Table 1.

Table 1. PCR Primers Used in Resequencing the IL13 Gene*.

Alias	Forward Primer (5'-3')	Primer Pos. *	Reverse primer (5'-3')	Primer Pos.*
IL13 Prom.1 †	CCTTGGTCCCTGGAGACCAC	85 - 104	CCATGTCGCCTTTTCCTGCT	1112 - 1131
IL13 Prom.2	GTTGAGCCCCATCGAGGAC	933 - 951	GGGTCTCACTATGTTGCCTGC	1448 - 1468
IL13 Prom.3	CCTAGGCAGGCAACATAGTG	1443 - 1462	GCTATGGGAATTTGGGGAGT	1722 - 1741
IL13 Prom.4	TTTAAGAGACTGGTTCATCG	1692 - 1711	ACTTATTGAGAAGGGTCCAG	1972 - 1991
IL13 Prom.5	TAAACCCACCCAGATCTTGG	1942 - 1961	TGGTCAACAAAAGCGCCATG	2213 - 2232
IL13EX1	GCTGCCACAAGACGCCAAGGCC	2134 - 2155	CCCTCATAGCTAGGACCCTGGC	2360 - 2381
IL13IN1.1	CCCTGTGCCTCCCTCTACAGCCC	2276 - 2298	GGAGAGCAGGTAGTCCCTGGGG	2558 - 2579
IL13IN1.2	CTGGGCTGGGGGGCTCAGC	2504 - 2522	CTCTACTAACGAATCCTCCTG	2744 - 2764
IL13IN1.3	GCAGCCATTCTCCCCAGCACC	2705 - 2725	CATGGACACTCTGGTGTTGGC	2946 - 2966
IL13IN1.4	GTGAGGTTAAGTGACAGAGG	2908 -2925	GTAAGGGACAAGCTGCATGC	3146 - 3165
IL13IN1.5	CCCGCAGGCCCCTGTCCTCCTG	3104 - 3125	GGTCTGCCCCAGCACAGGCC	3313 - 3332
IL13IN1.6	CTGCCAGGCCTGCCTCTGTG	3272 - 3291	CAGCTGTCAGGTTGATGCTC	3432 - 3451
IL13EX2	GCCAGCACTCTGCTCACTGTCAC	3367 - 3389	GCCCCATCCTCCCTGCACCC	3469 - 3488
IL13EX3	CCCAAGCAGGGCCTGACCCCTCGG	3669 - 3692	GCAGGGTGGGTGTGAGAGGG	3829 - 3848
IL13EX4.1	GGCGTTCTACTCACGTGCTGACC	4123 - 4145	GCTAAGGAATTTTACCCCTCCC	4377 - 4398
IL13EX4.2	GTCTTGGGTAGGCGGGAAGG	4350 - 4369	CCTGTGTGTGAAGTGGGTCC	4604 - 4623
IL13EX4.3	CCTTGCCAGACATGTGGTGGG	4579 - 4599	CGGATGAGGCTCCGAGGCC	4854 - 4872
IL13EX4.4	CTGCTACCTCACTGGGG	4831 - 4847	CCCCAAAGGCCAAAATGAAAGAC	5111 - 5133

* Genbank accession number U31120 was used as the reference sequence.
† To facilitate sequencing of this amplification product, the following forward and reverse nested primers pairs were used:
5'-TCTCCCGTTACATAAGGCCACC-3' (433-454) and 5'-CTGACTCCCAGAAGTCTGCCC-3' (759-779)
5'-AGCTTCGAGTGTGGACAGAGAGG-3' (800-822) and 5'-GTGGCCTTATGTAACGGGAGATG-3' (431-453)

Genotyping of IL13 polymorphisms

Three single nucleotide polymorphisms (SNPs) were genotyped in the IL13 gene using the following methods. SNP 3 (Arg130Gln) was genotyped in PCR-amplified genomic DNA by allelic discrimination using TaqMan™ technology (Perkin Elmer Applied Biosystems) on the ABI PRISM™ 7700 sequence detector (Perkin Elmer Applied Biosystems). Oligonucleotide probes homologous to the wildtype (5′-TCGCGAGGGACGGTTCAACTGAAA-3′; labelled with FAM 5′-reporter dye) and SNP (5′-TCGCGAGGGACAGTTCAACTGAAA-3′; labelled with TET 5′-reporter dye) se-

quences, and forward (5′-TAAAGGACCTGCTCTTACATTTAAAGAAA-3′) and reverse (5′-TCGAAAGCATCATTATTTGCAGAGACAGG-3′) PCR primers were designed using Primer Express™ (version 1.0; Perkin-Elmer Applied Biosystems) and synthesized by Perkin Elmer Applied Biosystems (Warrington, UK). Allelic discrimination reactions were carried out on 20 ng samples of genomic DNA in a 25 ml reaction containing 50-900 nM of each forward and reverse PCR primer, 50-200 nM of each FAM and TET probe, and 1X TaqMan® Universal PCR Master Mix (Perkin Elmer Applied Biosystems). PCR cycling conditions on the ABI PRISM™ 7700 were as follows: 50°C for 2 minutes; 95°C for 10 minutes; followed immediately by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

For SNP 6 (-1111 promoter) and SNP 7 (nt 4738, 3′-UTR), PCR was performed in a 10ml volume consisting of 60ng DNA, 0.4 mM each primer, 50mM KCl, 10mM Tris, 0.2mM each dNTP, 1.5 mM MgCl2, and 0.1 U Taq DNA polymerase. The promoter polymorphism was amplified using the primers 5′-ATGCCTTGTGAGGAGGGTCAC-3′ and 5′-CCAGTCTCGCAGGATCAACC-3′. The PCR products were then purified with Qiagen PCR purification kit and sequenced using the given primers with Big Dye kit and the 3700 DNA Analyzer (ABI). The sequence was performed in both directions and analyzed and viewed with Phred/Phrap[29], and Consed.[30] Genotypes were determined by visual inspection of the sequence files.
The 3′ UTR polymorphism was amplified using the primers 5′-CTTTGCTAACATATTTAATATTTAAATACG-3′ and 5′-GTCACCGTTGGGGATTGGGGAAG-3′. PCR cycling conditions were as follows: 94°C for 4 minutes, 30 cycles at 94°C for 30 seconds, 68°C 30 seconds and 72°C for 30 seconds with a final extension step of 72°C for 6 minutes. PCR products were digested with NheI (New England Biolabs) and the alleles resolved by electrophoresis on a 2% agarose gel. The fragment sizes were 289 bp for the G allele, and 252 bp and 37 bp for the A allele.

Genetic Analysis

Analysis was performed for 4 phenotypes including asthma and associated phenotypes: BHR, total serum IgE levels, and skin-test responsiveness. Genetic analysis was conducted with each of the biallelic polymorphisms by comparing differences of allele and genotype frequencies between cases and controls. For comparing the allele frequencies between cases and controls, chi-square tests were used. When genotypic frequencies were compared between cases and controls, chi-square tests assuming a dominant model were performed (due to the small number of homozygotes for the rare allele). No corrections were made for multiple comparisons for two reasons. First, since the phenotypes tested (asthma, BHR, total serum IgE levels, and skin test response) are strongly associated with each other in this population, the statistical analyses do not represent independent tests. Second, we performed tests for association with phenotypes that have been observed by other investigators, both to confirm previous results and to

better characterize asthma susceptibility in our population. As described previously, all of the probands fit published criteria for an asthma diagnosis.[27] For BHR, cases were defined as probands and spouses with a $PC_{20} \leq 32$ mg/ml histamine. The control group for the analysis of both "asthma" and BHR cases were BHR-negative spouses. Individuals were considered skin-test positive if one or more skin test showed a maximum wheal diameter of > 5mm. Individuals with high total serum IgE levels were defined as having total serum IgE > 100 IU/ml, since this value best distinguished individuals with high versus low levels after examining the overall frequency distribution for the group.[5,6] Total serum IgE was also analyzed as a quantitative trait following logarithm-transformation to approximate a normal distribution.

The linkage disequilibrium test between pairs of SNPs was based on an exact test assuming multinominal probability of the multilocus genotype, conditional on the single-locus genotype.[31] A Monte Carlo simulation was used to assess the significance, by permuting the single-locus genotypes among individuals in the sample to simulate the null distribution. The empirical p-values of the LD for each pair of SNPs was based on 10,000 replicate samples.

RESULTS

Characteristics of Population Sample

As illustrated in Table 2, the probands and spouses are of similar ages (mean = 52 and 51 years, respectively). All spouses are included in the table but only BHR-negative spouses ($PC_{20} > 32$ mg/ml histamine) were used for comparison with the probands for the asthma and hyperresponsiveness phenotypes. All probands and BHR (+) spouses were included in the BHR (+) group for the BHR comparison (with BHR (-) spouses). All probands were BHR (+) at the time of initial testing. One hundred and seventy-one of 184 were retested since 13 had an FEV_1 that was too low to be retested safely ($FEV_1 < 40\%$ predicted); 10% of those retested were no longer BHR (+). A high proportion of the probands (40.3%) are very hyperresponsive ($PC_{20} <$ 2 mg/ml). Although the probands were not selected for atopy, 80.9% had ≥ 1 positive skin-test compared with 29.9% of the spouses. The degree of overlap between the asthma and atopy phenotypes in this population is shown in Figure 1.

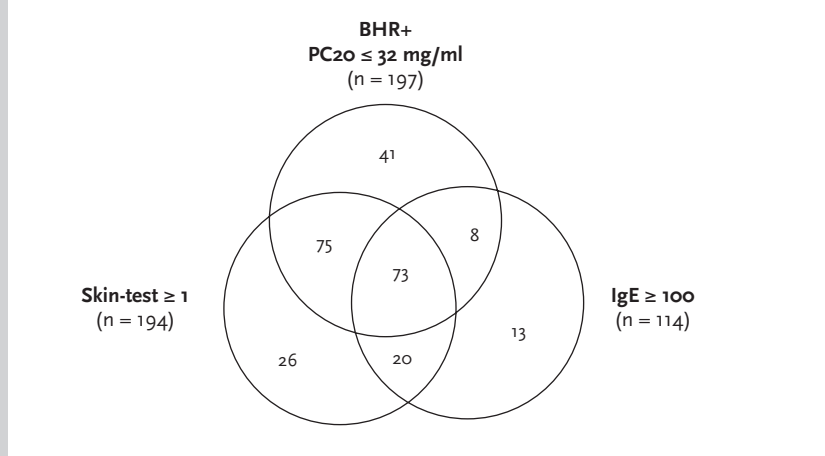
Resequencing of the IL13 gene to identify SNPs

The human IL13 gene is composed of 4 exons spanning 2.938 kb of genomic DNA .[32] SNPs in the IL13 gene were identified by resequencing genomic DNA from 20 probands and 20 controls (spouses) from the Dutch families. In addition to exons 1 to 4, over 2 kb of the 5'-flanking region and the first intron were resequenced because potential recognition sites for a number of transcription factors, interferon-inducible elements and enhancer elements have been localized to these regions .[33]

Table 2. Clinical Characteristics of Dutch Probands and Spouse

IL13 DATA	Probands	Spouses
Sex, M:F	114:70	70:114
Age, mean	52.1	51
SD	8.3	8.9
range	37-76	34-76
Total IgE, IU/ml (geometric mean)	93.0	26.5
% ≥ 100 IU/ml IgE	44.8	20.1
Skintest Response		
% with ≥ 1 positive skintest	80.9	29.9
% with ≥ 3 positive skintest	59.0	13.6
FEV_1 % Predicted Pre-Medication (mean)	69.1	98.7
FEV_1/FVC ratio (mean)	59.1	77.0
Reversibility (n)	181	184
%, ≥ 12% (baseline), (n)	66.3 (120)	12.5 (23)
%, ≥ 9% (predicted), (n)	63.5 (115)	16.8 (31)
Airway Obstruction		
% FEV_1/FVC ≤ 70% and FEV_1 ≤ 75%	52.7	2.2
PC_{20} (N)	171	183
PC_{20} > 32 mg/ml, %	9.9	75.4
PC_{20} ≤ 32 mg/ml, %	90	24.6
PC_{20} ≤ 16 mg/ml, %	81.2	16.4
PC_{20} ≤ 8 mg/ml, %	63.1	11.5
PC_{20} ≤ 4 mg/ml, %	55.5	6
PC_{20} ≤ 2 mg/ml, %	40.3	2.7
PC_{20} ≤ 1 mg/ml, %	22.8	0.0

Figure 1. Relationship of bronchial hyperrespnsiveness, skin-test response and total serum IgE levels in Dutch probands and spouses genotyped for IL13 polymorphisms.



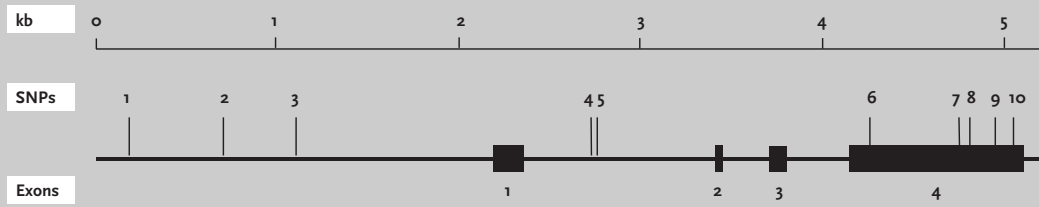


Figure 2 SNPs detected in IL13 gene

SNP	Nucleotide Position	Position Relative to +1 of ORF	Location	Allotype	Affected (n=20)	Unaffected (n=20)
1	175	-2039	5'-Promoter	CC	13	16
				CT	6	4
				TT	1	0
2	704	-1510†	5'-Promoter	AA	13	16
				AC	6	4
				CC	1	0
3	1103	-1111†	5'-Promoter	CC	15	16
				CT	4	4
				TT	1	0
4	2728	-	Intron 1	CC	16	14
				CA	2	5
				AA	1	1
5	2755	-	Intron 1	GG	17	16
				GC	2	4
				CC	0	0
6	4257	+389	Exon 4 (Arg130Gln)‡	GG	9	14
				GA	11	6
				AA	0	0
7	4738	+870	Exon 4 (3'-UTR)	GG	10	14
				GA	10	6
				AA	0	0
8	4793	+925	Exon 4 (3'-UTR)	CC	10	14
				CA	10	6
				AA	0	0
9	4962	+1094	Exon 4 (3'-UTR)	CC	10	13
				CT	10	6
				TT	0	0
10	5054	+1186	Exon 4 (3'-UTR)	TT	20	18
				TG	0	1

Figure 2. continued

SNPs Detected by resequencing of the IL13 Gene in this Dutch population. Eighteen overlapping PCR-amplified DNA fragments covering 2.214 kb of genomic DNA sequence 5' to +1 of the ORF, exons 1 to 4 (including intron-exon boundaries) and intron 1 of the IL13 gene were cycle sequenced in 20 affecteds (probands) and 20 unaffected individuals (spouses) as described in Materials and Methods. The allotype for each SNP was determined by visual inspection of the sequence traces. Top: Graphical representation of SNP locations within the IL13 gene. Bottom: Location of IL13 SNPs with allotype numbers and affection status of sequenced individuals.

* GenBank accession No. U31120 was used as the reference sequence. The coordinates for exons 1 thru 4 are nucleotides 2158-2345, 3403-3456, 3709-3813 and 4160-5095, respectively.
¶ 39 patients genotyped.
† The position of SNPs 2 and 3 relative to +1 differs between this report and Graves et al (2000) because GenBank accession number AC004039 was used as the reference sequence by the latter. The differences between U31120 and AC004039 are due to C insertions at positions 49963 and 49578 in AC004039 which are not present in U31120 (corresponding to positions 752-753 and 1136-1137, respectively).
‡ Corresponds to the Gln110Arg of Heinzmann et al. (2000) who use the amino acid coordinates of the mature protein (cytokine-web; http://www.psynix.co.uk/cytweb/targets/index.html)
UTR: untranslated region

Ten SNPs were identified by resequencing (Figure 2), with only one (SNP 6 located in exon 4) leading to a predicted amino acid change in the IL13 protein (glutamine for arginine amino acid substitution; Arg130Gln). Six of the SNPs have previously been identified in other population groups: SNP 3 (5'-promoter [14,16,34]); SNP 6 (exon 4[15-17]); SNP 2 (5'-promoter [16]); and SNPs 7, 8 and 9 (exon 4, 3'-untranslated region;[16]) SNPs 1 (5'-promoter), 4 and 5 (intron 1) and 10 (exon 4, 3'-untranslated region) are novel.

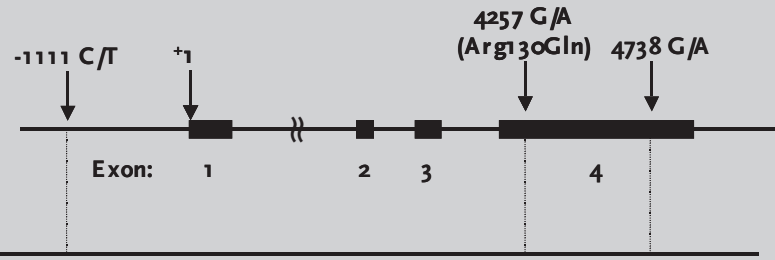
Association Analysis of IL13 with asthma and atopy phenotypes

One hundred and eighty four Dutch probands and spouses were genotyped and analyzed with SNPs 3, 6 and 7. These SNPs were located in the promoter region, exon four, and the 3' UTR (Figure 3). All SNPs were in Hardy-Weinberg equilibrium and the allele frequencies for each SNP are reported in Table 3. These frequencies are similar to those reported in other populations from the United States, Netherlands, and Germany [16], but differ from those from the United Kingdom and Japan .[17] Significant linkage disequilibrium was observed between the –1111 promoter and the 3' UTR SNP ($p<10^{-4}$) and between the Arg130Gln and 3' SNP ($p=10^{-4}$), but not between the –1111 promoter and the Arg130Gln.

Table 3. Allele Frequency in Dutch Proband/Spouse Population

	allele 1	allele 2
5' Promoter (SNP 3)	0.79	0.21
Arg130Gln (SNP 6)	0.75	0.25
3' UTR (SNP 7)	0.75	0.25

Figure 3 Location of SNPs genotyped in IL13 gene



This report:	Asthma/ ST†	Asthma/ ST†
Van der Pouw Kraan et al.:	Allergic Asthma	
Heinzmann et al.:		Asthma/ Allergic Asthma
Graves et al.:		Total serum IgE
Liu et al.:		Total serum IgE atopic dermatitis

†Skin test sensitivity

Schematic representation of the IL13 gene showing the genomic structure (boxes represent the 4 exons) and the location of SNPs genotyped in this study and others. Phenotypes with a significant association ($p<0.05$) are shown in the table below, aligned with the associated polymorphism.

The results of this study reveal significant associations between specific asthma and atopy phenotypes for the 5′ promoter and 3′ UTR SNPs (Table 4) The most significant associations were observed with the promoter polymorphism and the presence of both asthma and skin-test sensitivity (≥ 1 positive skin-test) to common aeroallergens. The promoter "T" allele was significantly more prevalent in the cases with asthma than in controls (24% versus 14%, p=0.004). This association was also present to a lesser degree in individuals with one or more positive skin-test (25% versus 17%) (p=0.01). When compared by genotype, the association with both asthma and allergy phenotypes was highly significant. TT homozygotes were much more common in cases than controls with regard to asthma (p=0.008), BHR (p=0.007), and asthma and skin-test sensitivity combined (p=0.006). While we did not observe a significant association of IL13 polymorphisms with IgE levels, there was evidence of higher total serum IgE levels with the rare allele of the promoter polymorphism (p=0.089; Table 5). When stratified by skin-test positive and skin-test negative individuals, we did not observe an association with total serum IgE levels in skin-test negative individuals, as previously reported with the Arg130Gln polymorphism.[16] Lower levels of significance were also observed with the 3′ UTR SNP (Table 4).

Table 4 Association between Asthma and Atopy Phenotypes and IL13 Polymorphisms in a Dutch Asthma Population

		Promoter			Arg130Gln			3'UTR		
		C	T	p-value	G	A	p-value	G	A	p-value
Asthma	Cases	261	81	**0.005**	230	74	n.s.	234	92	**0.043**
	Controls	204	34		178	62		184	48	
BHR	Cases	329	103	**0.003**	282	90	n.s.	294	110	0.066
	Controls	204	34		178	62		184	48	
Skin Test	Cases	289	89	**0.029**	240	84	n.s.	254	98	**0.046**
	Control	245	49		221	69		226	60	

†**Genotype**

		Promoter				Arg130Gln				3'UTR			
		CC	CT	TT	p-value	GG	GA	AA	p-value	GG	GA	AA	p-value
Asthma	Cases	99	63	9	**0.008**	89	52	11	n.s.	86	62	15	**0.02**
	Controls	87	30	2		67	44	9		77	30	9	
BHR	Cases	126	77	13	**0.007**	111	60	15	n.s.	109	76	17	n.s.
	Controls	87	30	2		67	44	9		77	30	9	
Skin Test	Cases	111	67	11	n.s.	89	62	11	n.s.	93	68	15	**0.02**
	Controls	102	41	4		89	43	13		94	38	11	
Asthma *and*	Cases	79	52	8	**0.006**	69	46	8	n.s.	66	53	13	**0.009**
Pos. skin-test	Controls	87	30	2		67	44	9		77	30	9	

*Analysis was performed using the number of each allele in cases and controls

†Analysis was performed using a dominant model for the genotypes in cases and controls

Table 5 Mean IgE levels for each genotype

Promoter	*Number*	*Mean Log [IgE] (± SD)*	*Geometric Mean total serum IgE (IU/ml)*	*p-value*
CC	213	1.63 (± 0.73)	42.66	n.s.
CT	108	1.79 (± 0.71)	61.66	
TT	15	1.87 (± 0.74)	74.13	
Arg130Gln				
GG	178	1.63 (± 0.69)	42.66	n.s.
GA	105	1.81 (± 0.72)	64.57	
AA	24	1.71 (± 0.75)	51.29	
3' UTR				
GG	187	1.63 (± 0.70)	42.66	n.s.
GA	106	1.82 (± 0.74)	66.07	
AA	26	1.77 (± 0.76)	58.88	

DISCUSSION

Asthma is an inflammatory airways disease characterized by bronchial hyperresponsiveness and airways obstruction. Atopy traits, such as elevated total serum IgE levels and positive allergen skin-test responses, are also associated with this disease and may predict the development of symptomatic asthma.[1,2,4] Interleukin 13 is expressed in asthmatic airways and has an important role in the production of IgE and is therefore an excellent biologic candidate gene for the development or expression of diseases with atopy components such as asthma.

In this study of the IL13 gene, 10 SNPs were detected of which 6 have been identified previously and 4 are novel. Of the former, SNPs 3 (-1111 promoter) and 6 (Arg130Gln) have been associated with increased risk of allergic asthma[14] and higher total serum IgE levels[16] or asthma[17], respectively. In addition, SNP 3 appears to promote increased binding of nuclear proteins to the promoter region[14], whereas the amino acid change resulting from SNP 6 could affect the interaction of IL13 with IL13Rα1.[16,17] In contrast, none of the remaining 8 SNPs occur within identified regulatory elements in the IL13 gene[e.g. 33], or alter the amino acid sequence, so their functional relevance is unclear at the present time. We investigated the contribution of SNPs 3, 6 and 7 using a case-control study in a Dutch asthma population consisting of probands, selected on the basis of a diagnosis of asthma, and their unaffected spouses. One SNP was chosen in the 3' UTR (SNP 7, nt 4738) to examine the potential regulatory elements in this region. These three SNPs encompassed the entire IL13 gene so that the contribution of genetic variation could be detected. Consistent with other reports that have evaluated IL13 polymorphisms, we observed a significant association of several polymorphisms in IL13 with various atopy or asthma phenotypes.

There are several unique properties of this study. The cases and controls are the parents in the same families used to identify linkage to chromosome 5q31, allowing us to examine candidate genes within this region in linked families. The comprehensive clinical data collected in these families have allowed us to examine multiple clinical phenotypes associated with both asthma and atopy. And finally, because of the study design, the controls were similar in regard to age and overall environmental exposures (allergens).
Both IL4 and IL13 can elevate baseline IgE levels. However, while an essential role for IL4 in the induction of asthma has been proposed, murine models have demonstrated the critical nature of IL13 independent of IL4. In a murine model of allergic airways hyperresponsiveness, blockade of IL13 reversed many of the characteristics found in allergic asthma such as airway hyperresponsiveness, eosinophil infiltration, and mucous production.[35] Furthermore, the effects of IL13 were shown to be mediated by a pathway dependent on the IL4Rα receptor.[35] Transgenic mice expressing IL13 specifically in the lungs exhibited increased BHR, bronchial eosinophilia, and increased mucous production.[36]
Using a mouse model for asthma, Symula et al.[37] were able to demonstrate the effects of quantitatively changing mouse IL4 and IL13 gene expression using transgenic mice constructed with human YACs from the chromosome 5q3 region. Surprisingly, the transgenic mice had significantly lower total IgE levels due to decreased endogenous gene expression, which influenced the development of Th2 cells. (Human IL4 and IL13 appear to have minimal activity in mice). Furthermore, when a BAC containing mouse IL4 and IL13 genes was transfected into the mice, a significant increase in IgE levels, BHR, and asthma was observed.[37]

It has been suggested that IL13 may be an important regulatory cytokine in the pathogenesis of asthma, while IL4Rα, which is required for the functioning of both IL4 and IL13, contributes primarily to atopy.[38] Several IL4Rα polymorphisms have been associated with a higher risk of atopy[19,21], atopic asthma[15], and variation in IgE levels.[21] In addition, specific alleles of these variants were shown to modulate the activity of IL4Rα.[19-21] In a recent study by Ober et al.[22] eight polymorphisms were studied in both inbred and outbred populations. Significant evidence for an association between these variants and the resulting haplotypes were observed for asthma and atopy. These studies indicate that biologic functions related to these disorders may involve this receptor.

The most significant associations with IL13 were identified in individuals with a diagnosis of asthma (i.e., the original probands in this study) and in individuals with the BHR phenotype (including affected spouses) (Table 4). The study by Heinzmann et al.[17] also supports this role of IL13 in asthma. In both the Japanese and the British populations, the most significant associations were observed between IL13 (Gln110Arg) and atopy and nonatopy asthma. There was no evidence of an association with this polymorphism and total serum IgE levels. In the Japanese population, strong asso-

ciation was observed with the Ile50Val IL4Rα polymorphism and IgE levels, both total serum and allergen-specific ($p<0.0001$). The coexistence of BHR and atopy characteristics (e.g., Figure 1) makes it difficult to discern the exact roles of IL4Rα and IL13. However, the interaction of these two genes as important components of IgE-mediated inflammatory responses supports the role of these cytokine pathways in the development or expression of atopy conditions and asthma.

Association analyses with specific polymorphisms in IL13 have produced varied results (Figure 3).[14-17] This may be due, in part, to the fact that each study is based on population samples that were ascertained differently. For example, the previous studies focused on recruitment of patients with allergic asthma[14,17], atopy[15], or random ascertainment from longitudinal and cross-sectional groups.[16] In our study design, we ascertained families based on asthma, but are also able to examine both asthma and atopy phenotypes. In addition, these families showed evidence for linkage to the region of chromosome 5q where IL13 is located.[5-7]

Another potential cause of differences in the results of these studies is that they were performed in different population groups. As linkage disequilibrium varies between populations, this would suggest one of two possibilities. First, it is possible that different polymorphisms or haplotypes within the IL13 gene contribute to the allergy phenotype in each population. Therefore, each analysis may be identifying the specific allele or haplotype responsible for the phenotype in that specific population. This suggests that several of the polymorphisms identified are capable of significantly altering the function of IL13, resulting in a predisposition to atopy, and reported differences are primarily due to the founder allele in that specific population. A second explanation is that an additional, unidentified sequence variant is responsible for the phenotype, and the level of detection (i.e. significance of the association) is dependent on the degree of linkage disequilibrium in that population for this region of chromosome 5q. In this case, the true susceptibility allele would have to be a fairly distant enhancer or promoter element, since IL13 resequencing studies have most likely identified all of the common variations within or near the gene (this report).[14,16] One candidate for such a distal element would be the conserved noncoding sequence (CNS-1) recently identified between IL13 and IL4.[39] This sequence is approximately 3kb from the 3' end of IL13 and 10kb from the 5' end of IL4, is highly conserved between mice, humans, cows, dogs, and rabbits (~80% identity), and was shown to specifically regulate IL4, IL13, and IL5 in human YAC transgenic mice. This region should be investigated to determine if polymorphisms are present that may influence susceptibility or expression of atopy or asthma. A variation in CNS-1 may explain the different results from the IL13 studies, and may also explain the conflicting results with a promoter polymorphism in IL4[13], since various degrees of linkage disequilibrium with CNS-1 and the IL13 promoter between populations may contribute to differences in reported associations.

Asthma and allergy are common diseases caused by an intricate interaction of genetic susceptibility and environmental exposure. This association study, as well as those reported by others, has implicated IL13 as a major component in the expression of these conditions. Functional studies examining the roles that these polymorphisms have on the activity or expression levels of IL13 and, perhaps more importantly, the downstream responses to these changes will provide valuable insight into the overall mechanisms that cause susceptibility to asthma and atopy. As we define specific genes that are associated with allergy and asthma phenotypes, patterns are beginning to develop that may be useful in delineating important biologic pathways, leading to a better understanding of gene-gene and gene-phenotype relationships in asthma and allergy. The importance of IL13 and its functional and genetic interactions with IL4 and IL4R in the development and expression asthma and atopy support this approach that combines molecular genetic techniques with clinical studies.

ACKNOWLEGMENTS

We would like to thank all participants of the study, and E. Gankema, H. Koops, M. Leever and D. Faber who assisted in the clinical testing. We are thankful to C.I.M. Panhuysen, B. Meijer, and G.G. Meijer, for their work in patient recruitment, and S. Zheng and O.C. Stine for assistance in the molecular laboratory.

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