

Multilocus haplotype analyses reveal association between 5 novel IL-15 polymorphisms and asthma

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Background: IL-15 is a T_H1-related cytokine that is involved in the inflammatory response in various infectious and autoimmune diseases. IL-15 has recently been shown to be upregulated in T-cell-mediated inflammatory disorders. The observations suggest a potential role for this cytokine in a variety of pathologic conditions, including T_H1-mediated and T_H2-mediated inflammatory diseases.

Objective: In this study, we searched for single nucleotide polymorphisms in the whole IL-15 gene and investigated their association with inflammatory and/or atopic phenotypes.

Methods: The screening for single nucleotide polymorphisms was performed by single-strand conformation polymorphism analysis. Genotyping of the identified polymorphisms was performed by restriction fragment length polymorphism. Genotypic association analysis used the Armitage trend test. Haplotype frequency estimation and subsequent testing for differences between cases and controls were performed by using the programs FASTEHPLUS and FAMHAP.

Results: We identified 5 novel noncoding nucleotide sequence variants, all of which were typed in our asthmatic, our atopic, and our control population. According to the Armitage trend test, none of the 5 polymorphisms is associated with the phenotype bronchial asthma or atopy. However, multilocus haplotype analysis based on simulations to find out whether the haplotype frequencies differed between cases and controls by using the program FAMHAP yielded a *P* value of 6.1×10^{-5} in the asthmatic versus the control population, which is highly significant. Furthermore, we obtained a nominally significant result of *P* = .0232 for the atopic versus the control population by using FAMHAP.

Conclusion: These results strongly underscore previous findings that suggest a potential role of this cytokine in allergic diseases. (J Allergy Clin Immunol 2004;113:896-901.)

Key words: Atopy, asthma, association, haplotype analysis, case-control analysis, inflammation, T_H1/T_H2

Allergic asthma is a chronic inflammatory disease associated with high serum IgE antibodies and local lung

Abbreviations used

EM: Expectation-maximization
HWE: Hardy-Weinberg equilibrium
SNP: Single nucleotide polymorphism

eosinophilia as a consequence of the function of several T-cell cytokines, especially IL-4, IL-5, and IL-13.¹⁻⁴ Cytokines play a pivotal role in the recruitment, proliferation, and survival of inflammatory cells in lung disorders. IL-15 is a cytokine with widespread mRNA expression in various tissues, including placenta, lung, and primarily monocytes in the peripheral blood.^{5,6} It promotes activation, proliferation, and cytokine release of various subsets of T cells,⁷⁻⁹ natural killer cells,¹⁰ mast cells,¹¹ and B cells.¹² The capability of IL-15 in inducing IFN- γ expression has been well documented.^{13,14} In healthy individuals, IL-15 has been shown to favor a T_H1 response by increasing the expression of IFN- γ .¹⁵ Aberrant IL-15 expression was observed in patients with inflammatory autoimmune disease, such as rheumatoid arthritis^{16,17} and inflammatory bowel disease,^{18,19} suggesting that IL-15 is involved in the pathogenesis of many inflammatory diseases. These findings suggest that IL-15 has the potential to skew the immunologic response in favor of a T_H1 rather than a T_H2 response. In contrast, the findings of Mori et al²⁰ that IL-15 promotes the production of IL-5 in human T_H cells and the fact that IL-15 has been shown to be a growth factor of mast cells¹¹ suggest a role for this cytokine in T_H2 immune response. All of these observations show a potential role for this cytokine in a variety of pathologic conditions, including T_H1-mediated and T_H2-mediated inflammatory disease.

The aim of our study was to search for single nucleotide polymorphisms (SNPs) in the whole IL-15 gene and to investigate their association with inflammatory and/or atopic phenotypes.

MATERIAL AND METHODS

Subjects

From July 2000 until January 2003, 321 children with suspected asthma (age 5 to 18 years) from southwestern Germany were recruited. All probands were characterized at University Children's Hospital by using a standardized clinical protocol. Asthma was

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diagnosed according to Thoracic Society criteria.²¹ The participants were asked to discontinue 1 week in advance any asthma or allergy medication before the clinical testing, which is a safe protocol in mild forms of asthma. The extended medical history was recorded, including occurrence and duration of wheezing symptoms, previous and acute medications, severity of previous asthma attacks, previous allergic rhinitis or conjunctivitis, atopic dermatitis, and family history of allergic diseases.

Skin prick test

Skin prick tests were performed by using intracutaneous applications of 17 common allergens, as well as positive (histamine) and negative controls. The following allergens were tested: house dust mites, different grass and tree pollens (grasses, rye, birch, hazel, mugwort), *Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium herbarum*, dog, cat, rabbit, duck, chicken, and horse dander. The diameter of the wheal responses was recorded after 15 minutes. The skin prick test was regarded as positive if the wheal of the allergen was at least half the size of the wheal of the positive control.

Specific and total IgE

Specific IgE was detected by ELISAs against mite allergens (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), different grass and tree pollens (grasses, rye, birch, hazel, mugwort), *A fumigatus*, *A alternata*, *C herbarum*, dog, cat, rabbit, duck, chicken, and horse dander (Magic Lite; Chiron Diagnostics, Fernwald, Germany). The cutoff point for a positive test was 1.43 Magic Lite units.²² Measurement of total serum IgE was performed by an enzyme allergosorbent test (Phadezym; Pharmacia, Uppsala, Sweden).

Pulmonary function tests

Pulmonary function tests were performed according to the protocols of the International Study of Asthma and Allergies in Childhood.²³ In addition, exercise-induced asthma was diagnosed by the following method. The probands performed physical exercise for 6 minutes under standardized conditions. The first spirometry and peak flow measurement was performed after 2 to 3 minutes and the second after 5 to 6 minutes. After 10 more minutes, the children inhaled with salbutamol, and a third spirometry and peak flow measurement was taken.

To test for bronchial hyperresponsiveness, inhalations with increasing concentrations of histamine were performed (0.13 mg/dL, 0.25 mg/dL, 0.5 mg/dL, 1 mg/dL, 2 mg/dL, 4 mg/dL, and 8 mg/dL). Testing was stopped after a 15% decrease in FEV₁.

Asthma definition

Two hundred thirty-one of the 321 recruited unrelated children from southwestern Germany were diagnosed as positive for bronchial asthma. The mean age was 10.8 years, and the male:female distribution was 2:1. The diagnosis was based on a clear-cut history of asthmatic symptoms, the use of antiasthmatic medication, and at least a degree of bronchial hyperreactivity. The antiasthmatic drugs included typical betamimetika like salbutamol and standard corticosteroids used in asthma treatment such as budesonide. Bronchial hyperresponsiveness was defined as a fall in FEV₁ by at least 15% in histamine testing or exercise provocation. Ninety-one of the 231 asthmatic individuals were mite-sensitized according to the following definition.

Mite-sensitive (atopic) definition

Specific IgE antibodies to highly purified crude extract of *D pteronyssinus* were detected by an ELISA (Magic Lite; ALK, Uppsala, Sweden). The cutoff point for a positive class 1 titer was

>1.43 ML units. The mite-sensitized (atopic) population consisted of 197 unrelated children. The individuals were recruited from the 321 children with suspected asthma described before and from a previous population described elsewhere.²⁴ All individuals were recruited from southwestern Germany. Ninety-one of the 197 mite-sensitized individuals had asthma according to the asthma definition.

Control population

Two hundred seventy unrelated randomly chosen probands were used as controls. All individuals were recruited from southwestern Germany. No medical history was taken, and no medical testing was performed on controls.

Single-strand conformation polymorphism analysis

The screening for SNPs was performed in a separate population of 50 unrelated white individuals recruited randomly from the same geographic area without regard to an asthmatic or atopic history.

DNA was extracted from peripheral blood leukocytes following standard protocols and column-purified (DNA Midikit; Qiagen, Cologne, Germany). After PCR, the products were resolved on nondenaturing polyacrylamide gels containing 10% or 0% glycerol at 20°C and 10°C for 2 hours.²⁵ The gels were silver-stained as described elsewhere.²⁶

Sequencing

Sequencing was performed by the dideoxy chain termination method²⁷ by using the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems, Darmstadt, Germany), with the same primers as the PCR. All of the following studies included reference individuals with known genotypes confirmed by sequencing.

Genotyping

Genotyping was performed by RFLP. The primers, PCR conditions, and restriction enzymes used are listed in Table I. All fragments were resolved on agarose gels.

The genotyping was performed by 2 investigators unaware of the phenotype.

Statistical analysis

An association analysis, based on the case-control design, was performed for each SNP by using the Armitage trend test, taking into account the individuals' genotypes rather than just the alleles, following the guidelines given by Sasieni²⁸ as implemented in the program FINETTI (Thomas F. Wienker, unpublished data; <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>, <http://ihg.gsf.de/linkage/download/finetti.zip>). Separate analyses were performed for the asthmatic versus the control population and for the atopic versus the control population. The Armitage trend test does not rely on the assumption of Hardy-Weinberg equilibrium (HWE) when testing for association between a marker and the disease of interest. Although deviation from HWE has been shown to inflate the chance of false-positive results when performing the allele-based test,²⁸ the Armitage trend test based on the genotypes remains valid even if HWE does not hold. Still, for each of the 5 SNPs, we tested whether the genotype distribution for each of the 3 populations was in HWE by using FINETTI. A significant deviation from HWE in controls could be a hint to population admixture. In addition to the analyses based on a single SNP, we performed haplotype frequency estimation, taking into account haplotypes of all 5 markers, and tested for differences between the case (ie, asthmatic or atopic) and control population by using the programs FASTEHPLUS²⁹ and FAMHAP (Genetic Epidemiology, 2004, Tim Becker and Michael Knapp - in press; <http://www.uni-bonn.de/~umt70e/becker.html>). Both programs implement

TABLE I. Primers, PCR conditions, and restriction enzymes for the RFLPs

Polymorphism	Primers	PCR conditions	Restriction enzyme
C267→T	5'-AAGGTAGGTGTTCAATAAGTA-3' 5'-TCCAGCAGCCATCCATCC TC-3'	58°C/35 cycles	TaqI
G367→A	5'-TCTTCAATACTTAAGGATTTAC-3' 5'-CAAAAGAGTGGGATAAGTGA-3'	53°C/35 cycles	MboII
A10504→G	5'-ATGTGCTCGGTGAGAAAAA-3' 5'-CAAAAAGTCAATCCAAATATTGTA-3'	54.5°C/35 cycles	RsaI
C13687→A	5'-ACATATTGTCCAAATGTTTCATCAA-3' 5'-CTGCCTTCATTCTAAGAGTTC-3'	60°C/35 cycles	DraI
A14035→T	5'-AGTTGCACTGATATTTTACCT-3' 5'-CAGTAGTCAGTGGTCCACTC-3'	56°C/35 cycles	DdeI

the expectation-maximization (EM) algorithm³⁰ to estimate the maximum-likelihood haplotype frequencies and perform a likelihood ratio test to find out whether they differ between cases and controls. In addition, the novel program FAMHAP performs simulations to obtain a *P* value that does not rely on asymptotic theory. This is particularly important when jointly looking at several markers, because there are many possible haplotypes in this case. Using the asymptotic χ^2 distribution of the likelihood ratio test statistic, with the corresponding high number of degrees of freedom, will most likely lead to a conservative test. This is because some of the possible haplotypes are not found in the sample, and thus the actual number of degrees of freedom is lower than the assumed number of all possible haplotypes minus 1. To determine the correct *P* value, FAMHAP permutes the disease status of the individuals for each simulated replicate such that the numbers of cases and controls are the same as in the original sample. Here, 1 million replicates were simulated for each *P* value. The crucial point is the re-estimation of the haplotype frequencies for each replicate and subsequent calculation of the likelihood ratio test statistic. The *P* value equals the fraction of replicates that yield a test statistic greater than or equal to the one obtained for the original sample. Clearly, this novel method as implemented in FAMHAP results in more accurate *P* values than those obtained with asymptotic theory. Also, FAMHAP yields more reliable results than simulation methods that estimate the haplotype frequencies only once for the original sample, assign the most likely haplotypes to each individual, and then resample these haplotypes between cases and controls. This is because FAMHAP does not artificially deduce haplotypes for single individuals (which necessarily leads to an incomplete and distorted picture) but relies solely on the estimation of haplotype frequencies and their differences between the 2 groups.

RESULTS

Identification of polymorphisms

Performing single-strand conformation polymorphism analysis, we found the following 5 variants in the IL-15 gene (numbers refer to the sequence of accession number X91233): C267→T, G367→A, A10504→G, C13687→A, and A14035→T (Table II).

Meanwhile, 3 of the 5 variants could be confirmed in the course of the human genome project. The variant C267→T corresponds to rs2254514, the variant G367→A to rs2857261, and the variant A14035→T to rs1057972.

Genotyping of the polymorphisms

We genotyped the 5 polymorphisms in the 231 asthmatic individuals, in the 197 mite-sensitized individuals,

and in the 270 control individuals. The allele frequencies of the polymorphisms are shown in Table II. The results of the association analysis with the Armitage trend test for the asthmatic and the atopic population are listed in Table III. None of the 5 polymorphisms showed significant association with bronchial asthma or with atopy.

The results of the HWE calculations for the asthmatic, the atopic, and the control population are listed in Table IV. All polymorphisms were in HWE with the exception of the polymorphism A10504→G. This polymorphism showed a heterozygote deficit only in the control population, with a *P* value of .01. Table V shows the frequencies estimated by FAMHAP in the populations of asthmatic and atopic individuals and in the control population for haplotypes that occurred with a frequency of at least 0.005 in either population.

The likelihood ratio test of association performed by FASTEHPPLUS with the 5 marker haplotypes for the atopic versus the control population, which relies on asymptotic theory, resulted in a *P* value of .4656; the test for the asthmatic versus the control population yielded a *P* value of .0443 (χ^2 with 31 *df*, upper line of first column of Table VI). The corresponding values of the likelihood ratio test statistic were 45.57 and 31.01, respectively. When taking into account that only 15 and 16 of all 32 possible haplotypes occurred in the case-control samples for asthma and atopy, the corrected *P* values were 3.29×10^{-5} and .0087, respectively (χ^2 with 14 and 15 *df*, lower line of first column of Table VI). The simulation-based test performed by FAMHAP (second column of Table VI) for the 5 marker haplotypes resulted in *P* values of .0232 and 6.1×10^{-5} for the atopic and asthmatic versus the control population, respectively.

In summary, both FASTEHPPLUS and FAMHAP found significantly different haplotype frequencies for the asthmatic and the control population, but only FAMHAP found a nominally significant difference between the atopic and the control population, if one did not adjust the number of degrees of freedom assumed by FASTEHPPLUS.

DISCUSSION

Genetic association analysis involving SNPs and haplotypes has become increasingly important for the

TABLE II. Identified polymorphisms, positions, and allele frequencies (wild-type allele) within the gene coding for IL-15

Polymorphism	Gene position	Frequency, asthmatic subjects	Frequency, atopic subjects	Frequency, controls
C267→T	80 bp in front of the start codon	0.72	0.73	0.71
G367→A	First intron	0.51	0.48	0.47
A10504→G	98 bp before the 5th exon	0.89	0.89	0.86
C13687→A	Exon 6, 83 bp behind the stop codon	0.88	0.87	0.88
A14035→T	3' end	0.50	0.54	0.55

TABLE III. Genotype distributions (homozygous—wild-type, heterozygous, homozygous-mutant) as well as results of the association analyses with the Armitage trend test for the asthmatic versus control population and the atopic versus control population

Polymorphism	Genotypes		Armitage trend test <i>P</i> value	Genotypes		Armitage trend test <i>P</i> value
	Asthma	Controls		Atopy	Controls	
C267→T	131;87;13	132;114;22	<i>P</i> = .07	99;83;11	132;114;22	<i>P</i> = .43
G367→A	60;114;57	55;141;73	<i>P</i> = .20	40;106;49	55;141;73	<i>P</i> = .74
A10504→G	182;41;6	203;56;10	<i>P</i> = .26	153;40;3	203;56;10	<i>P</i> = .33
C13687→A	173;56;2	204;64;1	<i>P</i> = .72	146;47;3	204;64;1	<i>P</i> = .55
A14035→T	58;112;61	78;138;53	<i>P</i> = .09	51;106;39	78;138;53	<i>P</i> = .62

TABLE IV. Results of the HWE calculations for the asthmatic, atopic, and control populations obtained with FINETTI

Polymorphism	HWE test/FINETTI		
	Asthma	Atopy	Control
C267→T	<i>P</i> = .77	<i>P</i> = .23	<i>P</i> = .70
G367→A	<i>P</i> = .84	<i>P</i> = .21	<i>P</i> = .38
A10504→G	<i>P</i> = .09	<i>P</i> = .73	<i>P</i> = .01
C13687→A	<i>P</i> = .38	<i>P</i> = 1.00	<i>P</i> = .14
A14035→T	<i>P</i> = .64	<i>P</i> = .23	<i>P</i> = .56

study of human disease. Haplotype analysis, which describes the patterns of linkage disequilibrium across a region, can provide important information concerning the contribution of a gene to a specific disease.³¹ In the context of case-control association studies with haplotypes, whether haplotype frequencies differ between the 2 groups is tested. Most of these tests provide a global significance for the simultaneous comparison of all haplotypes. Computational haplotyping methods³² and implementations of the EM algorithm³⁰ have been found to perform well. Comparison of haplotype frequencies based on physically haplotyped, phase-known data with haplotype frequency estimates obtained by using the EM algorithm shows that they do not differ significantly.^{33,34} Our single-marker tests for association using the Armitage trend test did not yield significant results for any of the 5 analyzed SNPs. On the other hand, we found significant results for the 5 marker haplotype analyses of the asthma phenotype with both FASTERPLUS²⁹ and the new program FAMHAP. Interestingly, in a recent study,³⁵ haplotype analyses could increase the significance in comparison with single-marker association analyses. An explanation for the fact that we obtained significant results only for the multilocus haplotype analyses could be that

TABLE V. Results of the haplotype frequency estimation for the bronchial asthma, atopic, and control populations obtained with FAMHAP*

Haplotypes	Asthmatic subjects	Controls	Atopic subjects
1 1 1 1 1	0.011018	0.011367	0.013065
1 1 1 1 2	0.493282	0.443160	0.460933
1 1 1 2 1	0.002251	0.000000	0.005276
1 1 2 1 1	0.000000	0.001742	0.000000
1 2 1 1 1	0.017813	0.000000	0.008102
1 2 1 1 2	0.002225	0.000000	0.002455
1 2 1 2 1	0.124848	0.114065	0.126155
1 2 2 1 1	0.100117	0.133786	0.103264
1 2 2 1 2	0.000000	0.000000	0.002854
1 2 2 2 1	0.003906	0.000000	0.005874
2 1 1 1 2	0.000000	0.008246	0.000000
2 1 2 1 2	0.000000	0.001778	0.000000
2 2 1 1 1	0.221801	0.273183	0.259581
2 2 1 1 2	0.011043	0.000000	0.005260
2 2 1 2 1	0.000000	0.009530	0.000000
2 2 2 1 1	0.011698	0.003143	0.007179

*Alleles: 1, wild-type; 2, mutation.

only the SNPs C267→T and A14035→T show borderline significance, and that the combination of the information of all SNPs increases the sensitivity, and therefore the significance, as found by Schwab et al.³⁵

It is important to note that we found strong differences between the uncorrected *P* values obtained by the program FASTERPLUS, which relies on asymptotic theory (χ^2 with 31 *df*), and the simulated *P* values yielded by FAMHAP. FASTERPLUS obtains higher *P* values (ie, less significant) than FAMHAP for both asthma (*P* = .0443 vs 6.1×10^{-5}) and atopy (*P* = .4656 vs .0232). As pointed out in the section Statistical Analysis, this is because taking the *P* value from the χ^2 distribution with

TABLE VI. Results of the test for differences between the estimated haplotype frequencies for cases and controls with FASTEHPPLUS and FAMHAP*

FASTEHPPLUS		FAMHAP	
Asthma	Atopy	Asthma	Atopy
$P = .0443$ (31 df)	$P = .4655$ (31 df)		
$P = 3.29 \times 10^{-5}$ (14 df)	$P = .0087$ (15 df)	$P = 6.1 \times 10^{-5}$	$P = .0232$

*The upper line of the first column shows the P values obtained by FASTEHPPLUS, assuming a χ^2 distribution with 31 degrees of freedom. P values are shown in the lower line for the number of degrees of freedom adjusted to the number of actually occurring haplotypes minus 1. The simulated P values obtained with FAMHAP are given in the second column.

the number of degrees of freedom corresponding to the number of all possible haplotypes minus 1, as performed by FASTEHPPLUS, often leads to a conservative test, because some of the possible haplotypes may not be found in the sample. In fact, only 15 and 16 of the 32 possible haplotypes actually occur in the case-control sample for asthma and atopy, respectively. If the values of the likelihood ratio test statistic for asthma and atopy, 45.57 and 31.01, are compared with a χ^2 distribution with 14 and 15 degrees of freedom, the resulting P values are 3.3×10^{-5} and .0088, respectively, which are much closer to the values obtained by FAMHAP. It should be emphasized that FAMHAP permutes the disease status and re-estimates haplotype frequencies for each simulated replicate. Therefore, it yields more accurate P values than methods that perform haplotype frequency estimation only once, assign haplotypes to the individuals, and then permute these haplotypes (see the Statistical Analysis section).

Recent studies showed that (1) IL-15 may play a role in the initiation of T_H2 -type immune responses by inducing IL-4 production from mast cells,³⁶ (2) IL-15 may perpetuate allergic inflammation by reduction of spontaneous eosinophil apoptosis,³⁷ and (3) IL-15 levels were increased and IL-13 levels were decreased in sputum fluid from steroid-treated compared with non-steroid-treated asthmatics.

Our finding is therefore in accordance with previous findings that IL-15 may have an immunoregulatory role in asthma.

In conclusion, the aim of our study was to search for SNPs in the whole IL-15 gene and to investigate their association with inflammatory and/or atopic phenotypes. Altogether, based on haplotype analysis, we could show that the analyzed 5 novel IL-15 polymorphisms are strongly associated with the asthmatic phenotype, and possibly with the atopic phenotype as well.

Approval

The collection of serum and DNA material as well as the experiments were approved by the Ethical Commission of the University of Freiburg. A statement of informed consent was signed by all participants or their parents.

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