

Original article

Confirmation of association of *IL-15* with pediatric asthma and comparison of different controls

Background: Interleukin (IL)-15 is an important mediator in chronic inflammatory diseases. Recently, we have described the association of *IL-15* haplotypes with bronchial asthma. Asthma genetics is highly complex – about every second candidate gene is not confirmed in consecutive studies. We were interested in whether association of asthma with *IL-15* holds in a second population. Furthermore, we sought to investigate the effect of different controls.

Methods: Five *IL-15* polymorphisms were genotyped on the German Multi-center Allergy Study (MAS) cohort consisting of 886 children who were followed up from birth to 10 years of age. At 10 years of age, 96 were found to be asthmatic. MAS children who never had any wheezing symptoms ($n = 576$), who were never diagnosed with asthma ($n = 790$) and 129 super controls who had never had any atopic disorder were used as controls. Finally, 270 randomly chosen adults served as controls.

Results: Association was confirmed with single polymorphism and haplotypes. The super controls showed the highest difference to the asthmatics regarding haplotype frequencies. However, the effect escaped statistical significance, most likely because of the small sample size.

Conclusion: Association of *IL-15* with asthma was confirmed. Although super controls might be the most suitable, more numbers are needed. This might hamper the value of these controls especially when investigating common diseases.

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Bronchial asthma is a chronic inflammatory disease, based on an inappropriate activation of the immune system by environmental aeroallergens and other stimuli (1). It is associated with bronchial hyperreactivity and local lung eosinophilia, and is present in children mostly with high serum IgE levels (2). During the allergic response, several cytokines, especially interleukin (IL)-4, IL-5 and IL-13 are released by CD4⁺ T-helper (TH) 2 lymphocytes. IL-4 and IL-13 play a key role in IgE-isotype switching and therefore in the genesis of the allergic cascade leading to persistent airway inflammation (3, 4).

In contrast, IL-2, interferon (IFN)- γ and tumor necrosis factor (TNF)- α are typical mediators of the TH1 immune response which are important in the induction of cell-mediated immunity and in the inhibition of TH2 immune responses (5, 6). Thus, cytokines of the TH1-immune response are supposed to suppress TH2-mediated allergic responses.

Interleukin-15 is a TH1-related cytokine that regulates the activation, proliferation and cytokine release of T cells, natural killer cells, mast cells and B cells (7–10). It promotes activation and migration of T cells (11) as well

as cytokine release (12). IL-15 shares many biological activities with IL-2, but opposite effects have also been described (13). During signaling, both interact with the β - and γ -chain of the IL-2 receptor; in addition, IL-15 has a unique α -chain (14).

Investigations on IL-15 expression in patients with inflammatory autoimmune diseases like rheumatoid arthritis (15, 16), inflammatory bowel diseases (17, 18), sarcoidosis (19) and chronic active hepatitis (20) suggest that IL-15 is involved in the pathogenesis of inflammatory disorders. Besides, studies in mice showed that inhibition of IL-15 prevents the induction of allergen-specific T-cells and allergic inflammation (21). Thus IL-15 may influence both TH1- and TH2-related diseases (22).

Recently, we have described an association of haplotypes based on five *IL-15* polymorphisms with pediatric asthma (23). The genetic basis of bronchial asthma is highly complex and therefore the results of association analyses in different study populations are often conflicting. This is also reflected by the fact that only every second asthma gene described in one population has been confirmed in a second independent sample (24). In part,

this might be due to the occurrence of type 1 errors (false-positive findings). Other reasons for conflicting results are different ethnic background of the study populations, variable sample sizes and the selection criteria for cases. Moreover, the characterization and recruitment of controls might be crucial for association analyses.

Thus, the aim of this study was to confirm the association of *IL-15* haplotypes with asthma in a second independent population of German asthmatic children. Furthermore, we wanted to investigate the effects of different selected controls on association results.

Materials and methods

The MAS cohort

The German Multicenter Allergy Study (MAS) has been described in detail before (25). Briefly, MAS initially comprised 1314 children of German descent born in the year 1990 who were followed up from birth to the age of 10 years. Yearly follow-up visits included standardized interviews, questionnaires and physical examinations. Total and specific IgE antibodies against nine common food and inhalant allergens (CAP-radio-allergo-sorbent-assay fluoro enzyme immuno assay [RAST FEIA]; Pharmacia, Uppsala, Sweden) were determined at the age of 1, 2, 3, 5, 7 and 10 years. Genomic DNA from blood samples was obtained in 886 MAS children. Current asthma at age 10 years was defined when one or more wheezing episodes had been reported during the previous 12 months; 96 children fulfilled this criteria.

Control groups of the MAS cohort

- 1 Non-atopic, non-asthmatic control subjects ($n = 129$) were also selected from the MAS cohort. They had no symptoms of asthma, allergic rhinitis or atopic dermatitis ever [for definition of phenotypes, see Nickel *et al.* (26)] and IgE against any tested allergen was below the detection limit (< 0.35 kU/l) at the age of 7 and 10 years.
- 2 The second control group comprised MAS children who never had any wheezing symptoms and were never diagnosed as having asthma ($n = 576$).
- 3 Finally, all children who had no diagnosis of asthma ever were included in a third control group ($n = 790$).

Adult control population

A total of 270 German adults were randomly chosen for this control population (aged 19–40 years, mean age 24.6 years). No medical history was elevated and no medical testing was performed on these controls. They were already part of the initial association study (23), but were included in this study also in order to compare the effect of different control populations on association results.

Sequencing

For each polymorphism, three controls (homozygous wildtype, heterozygous and homozygous mutation) were sequenced by the dideoxy chain termination method (27) using the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems, Darmstadt, Germany). All the following studies included these three reference individuals for each polymorphism.

Genotyping

DNA was extracted from peripheral blood leukocytes following standard protocols and purified column (DNA midikit, Qiagen, Hilden, Germany). All five polymorphisms were typed by means of restriction fragment-length polymorphisms as described previously (23). The numbering of *IL-15* polymorphisms was based on the sequence of accession number X91233.

Statistical analysis

An association analysis was performed for each polymorphism using Armitage's trend test. This test takes into account the individuals' genotypes rather than just the alleles, following the guidelines given by Sasieni (28) as implemented in the program FINETTI (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl> and <http://ihg.gsf.de/linkage/download/finetti.zip>). Separate analyses were performed for the asthmatic and the different control populations. Armitage's trend test does not rely on the assumption of Hardy–Weinberg equilibrium (HWE) when testing for association. Thus Armitage's trend test based on the genotypes remains valid even if HWE does not hold. Still, HWE was calculated for each polymorphism in the populations also using the program FINETTI.

In addition to analyses based on single polymorphisms, we performed haplotype frequency estimation, taking into account haplotypes of all five polymorphisms, and tested for differences between the asthmatic and control populations by using the program FAMHAP (29). Using the program FAMHAP, the expectation–maximization (EM) algorithm to estimate the maximum-likelihood haplotype frequencies is implemented, and a likelihood-ratio test to find out whether the frequencies differ between cases and controls is performed. In addition, simulations are performed to obtain a *P*-value which does not rely on asymptotic theory. Therefore, 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, 1 000 000 replicates were simulated for each *P*-value. The *P*-value given by FAMHAP equals the fraction of replicates which yield a test statistic greater than or equal to the one obtained for the original sample.

Approval

The collection of blood and the experimental procedures were approved by the Ethical Committee of the University of Berlin and Freiburg, respectively. A statement of informed consent was signed by all participants or, in the case of children signed by their parents.

Results

Genotyping

We genotyped all polymorphisms within the promotor and coding region of *IL-15* which were identified by single strand conformation polymorphism (SSCP) analyses and sequencing in the initial study. Meanwhile, all five polymorphisms have also been identified by the human genome project. The location of the polymorphisms within the gene and the corresponding rs numbers are given in Table 1.

The polymorphisms of the 886 children of the MAS cohort were typed. The HWE in the different populations is also given in Table 1. All polymorphisms were in HWE.

Table 1. Frequency of the five polymorphisms under investigation and results of Hardy–Weinberg equilibrium for the asthmatic and the four different control populations obtained with the program FINETTI

SNP	rs number	Allelic frequency					HWE				
		Asthma	Super controls	Never wheeze	Never asthma	Adult controls	Asthma	Super controls	Never wheeze	Never asthma	Adult controls
C267T	rs2254514	0.76	0.68	0.72	0.71	0.71	0.84	0.59	0.14	0.29	0.70
G367A	rs2857261	0.51	0.47	0.48	0.48	0.47	0.54	0.44	0.55	0.98	0.38
A10504G	rs7349640	0.88	0.87	0.85	0.85	0.86	1.00	0.69	0.59	0.86	0.01
C13687A	rs10519613	0.84	0.91	0.90	0.89	0.88	0.72	0.27	0.30	0.30	0.14
A14035T	rs1057972	0.50	0.54	0.54	0.53	0.55	0.54	0.47	0.81	0.91	0.56

Table 2. *P*-values for association with asthma using the different control populations by Armitage's trend test

SNP	Asthma–super controls	Asthma–never wheeze	Asthma–never asthma	Asthma–adult controls
rs2254514	0.066	0.296	0.168	0.1405
rs2857261	0.492	0.476	0.504	0.3436
rs7349640	0.923	0.270	0.409	0.5910
rs10519613	0.025	0.015	0.015	0.1262
rs1057972	0.307	0.243	0.289	0.2085

Table 3. Results of the haplotype analyses using the program FAMHAP. Again the four different control populations were used

	<i>P</i> -value
Asthma–super controls	0.086
Asthma–never wheeze	0.079
Asthma–never asthma	0.074
Asthma–adult controls	0.034

In order to allow an easy comparison of the different control populations, we also included the allelic frequency and HWE of the previously genotyped adult control population in this table (23). In this population, all polymorphisms but rs7349640 were in HWE.

Association analysis

The results of the association analyses by the Armitage's trend test are given in Table 2. The polymorphism rs10519613 showed an association with bronchial asthma using all control populations except the adult controls. The other polymorphisms showed no association with asthma.

Haplotype analyses

The results of the haplotype analyses obtained by FAMHAP are listed in Table 3. A significant difference in haplotype distribution between asthmatic children and controls was only seen using the adult control population ($P = 0.034$). However, the *P*-values using the other control popula-

Table 4. Results of the haplotype frequency estimation obtained by FAMHAP

	Asthma	Super controls	Never wheeze	Never asthma	Adult controls
1 1 1 1 1		0.0212	0.0190	0.0174	0.0113
1 1 1 1 2	0.4777	0.4264	0.4369	0.4402	0.4434
1 1 1 2 1	0.0056	0.0124	0.0035	0.0024	
1 1 1 2 2	0.0056		0.0120	0.0104	
1 2 1 1 1	0.0056	0.0083	0.0111	0.0095	
1 2 1 2 1	0.1450	0.0617	0.0840	0.0848	0.1136
1 2 2 1 1	0.1000	0.1222	0.1468	0.1374	0.1352
1 2 2 1 2	0.0167		0.0043	0.0027	
2 1 1 1 2	0.0056	0.0082	0.0115	0.0049	0.0082
2 2 1 1 1	0.2277	0.3055	0.2672	0.2744	0.2722

Each number indicate the allele of one SNP: 1 = wildtype, 2 = mutation. The SNPs are listed in the order of their occurrence in the gene. Shown are haplotypes with a frequency ≥ 0.01 in at least one population. The common haplotypes are shown in bold.

tions differed only slightly and missed significance marginally. Thus, it is not possible to decide which controls show the most pronounced difference compared with the asthmatic population just on the basis of *P*-values for association.

In Table 4, the haplotypes with a frequency of at least 0.01 in either population are listed. Four high-frequency haplotypes exist in all populations. The group of children who never had an atopic disease showed the greatest difference in haplotype frequencies compared with the asthmatics for all but one haplotype. These super controls are – with respect to the difference in haplotype frequencies – followed by those children who never had any wheezing symptoms, then the children who were never diagnosed as having asthma and finally the adult controls –showing the smallest effect.

Discussion

The primary aim of the study was to test whether association of *IL-15* with asthma holds in a second independent population of German asthmatic children. For this purpose, we chose the MAS cohort, which consists of 96 asthmatics out of 886 children. Secondly,

we wanted to utilize the extensive clinical characterization of the MAS cohort by building up distinguished control populations and measuring their possible effect on association results. Until now, there is still much speculation about the make-up of the most suitable controls for case-control association studies. However, to our knowledge, no systematic comparison of different control populations has been conducted so far.

We chose the following inclusion criteria for the control groups: the first group consists of children who never had any atopic disease, that is no atopic dermatitis, rhinitis or asthma. Furthermore, they never had any wheezing symptoms and IgE levels to common allergens were found to be negative at the age of 7 and 10 years. This group should represent the ideal controls and thus the term 'super controls'.

Secondly, the 'never wheeze' group comprises children who have a negative history for wheezing and no diagnosis of asthma ever. Therefore, atopic children were not excluded from this control group.

Thirdly, a group of children who were never diagnosed as having asthma were used. Again, this population included children who were atopic or had suffered from transient wheezing in infancy. Finally, we used an adult control population; here, no medical history was taken and no medical testing was performed. Thus, this population might well include about 10% of asthmatics.

The association of single polymorphisms with asthma was quite weak regardless of the control population. In fact, only one polymorphism was found to be significantly associated (rs10519613) with all control populations except the adult controls. This in accordance to the initial association study in which association was mainly seen with haplotypes but not with any single polymorphisms (23).

Haplotype analyses confirmed the association with asthma, too, but again the effect was quite weak. Most likely, this is due to the small number of asthmatic children in this study ($n = 96$). As a strong association of exactly the same haplotypes have already been shown in a larger population of asthmatic children, we think that the association is true and not caused by a type I error. Moreover, one could also argue that – when association is already detected in a small population – the effect of the polymorphisms on the genetics of asthma should be quite strong.

Regarding the different control populations, no major effect was seen on the P -values for association of single

polymorphisms or haplotypes. However, when looking at the allelic frequency of single polymorphisms and, in particular, the haplotype frequency, a distinct trend can be seen.

In fact, the non-asthmatic, non-atopic controls showed the most pronounced difference in haplotype frequency to the asthmatics for all but one major haplotype. This might escape statistical significance because of the small sample size of this control group ($n = 129$). It is followed by the never wheezing group, the never asthma group and finally by the adult control population. Thus, the discrepancy in haplotype distribution becomes smaller the more likely asthmatics are included in the control population. This observation is clearly in favor of the utilization of most strictly defined controls in case-control studies. However, most likely, the effect will reach statistical significance only when the number of these control subjects is quite large. This might cause problems in the recruitment of a sufficient number of controls, especially in the context of atopic diseases. Nowadays, about 40% of children have at least one atopic disorder and many children have at least one episode of wheezing during infancy.

In conclusion, we were able to replicate the association of *IL-15* with bronchial asthma in a second independent population of asthmatic children. The analyses of the allele and haplotype frequencies might approve the expectations that super controls are the most suitable control population. However, this effect will probably only reach statistical significance when investigating a large number of strictly defined controls. This might hamper the value of these controls. In addition, more studies on different genes are needed before final conclusions can be drawn.

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