Polymorphisms and Haplotypes of Acid Mammalian Chitinase Are Associated with Bronchial Asthma

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Rationale: **Chitinases are enzymes that cleave chitin, a polysaccharide contained in many parasites of humans. Recent studies in mouse models of bronchial asthma have shown that acid mammalian chitinase (AMCase) is involved in the pathophysiology of asthma. It acts downstream of interleukin-13; inhibition of AMCase leads to an abrogated T-helper cell 2 inflammation, less bronchial hyperreactivity, and fewer eosinophils.**

Objectives: **The aim of this study was to identify common genetic variants in human *AMCase* and to use them to test for association of *AMCase* with pediatric asthma.**

Methods: **By sequencing the promotor region and all 11 exons on 30 individuals, 12 high-frequency polymorphisms were identified. Genotyping of six variants in exons and one promotor polymorphism was performed on the following populations by means of restriction fragment length polymorphisms: 322 children with asthma, 270 randomly chosen adult controls, and a pediatric control population consisting of 565 children who, at age 10 yr, had never wheezed and never been diagnosed having asthma.**

Measurements and Main Results: **We identified three known and two new amino acid variants. Analyses by the Armitage's trend test using both control populations showed association of the newly identified variant K17R and the nearby noncoding polymorphism rs3818822 with asthma ($p = 0.0031$ and $p = 0.0003$, respectively). In addition, haplotype analyses revealed strong association of haplotypes with the disease (asthma population vs. pediatric control subjects, $p < 10^{-10}$).**

Conclusions: **This newly described association between *AMCase* polymorphisms and asthma adds further evidence supporting the involvement of AMCase in the development of asthma.**

Keywords: asthma; genetic; pediatric

Chitin represents the second most abundant polysaccharide on earth, next to cellulose. It is expressed in the wall of fungi, in the microfilarial sheath of parasitic nematodes, and the exoskeleton of all types of arthropods. Chitin can be degraded by special enzymes—the so-called chitinases. Although these enzymes are highly conserved between species during the course of evolution (1), it was believed for a long time that chitinases had no function in humans because it was assumed that humans completely lack endogenous chitin and endogenous substrates for chitinases. Only in recent years has it become evident that chitinases also exist in humans and more has been learned about their role in human diseases. The first identified chitinase is called chitotriosidase (2). Chitotriosidase has gained attention in the context of Gaucher disease, in which strong elevation of chitotriosidase activity can be measured in plasma (3).

Some years ago, a second chitinase was identified (4). This enzyme has a pH optimum at around 2 and is therefore called acid mammalian chitinase (AMCase). Only recently, Zhu and colleagues have observed the important function of AMCase in the development of bronchial asthma in a mice model (5). AMCase is highly expressed in lungs of mice sensitized to ovalbumin. Inhibition of AMCase leads to an abrogated T-helper cell type 2 inflammation, to reduced bronchial hyperreactivity, and lower eosinophil counts. Furthermore, it was demonstrated that AMCase acts downstream of interleukin-13. Similar results were obtained with two other chitinase homologous proteins in mice models (6). A gene expression study in two different mouse models of asthma found AMCase highly expressed in the asthmatic phenotype (7).

In humans, AMCase is highly expressed in lungs of patients with asthma, but not in healthy lungs (5). Another study found that expression of AMCase mainly occurred in alveolar macrophages and the gastrointestinal tract, whereas chitotriosidase was only expressed by phagocytes (8).

Two intriguing studies have suggested that chitin might protect from asthma: mice given orally small amounts of chitin before and during allergen immunization showed decreased IgE levels and lung eosinophils compared with mice not given chitin (9). In addition, the intranasal application of chitin reduces the allergic hypersensitivity to house dust mite antigen and *Aspergillus fumigatus* in mice (10).

So far, not much is known about the function of chitinases in humans. As the enzyme cleaves chitin, it might protect humans against the infections by the previously mentioned pathogens as already shown in mice models (11). The possible involvement of AMCase in the development of bronchial asthma might thus be particularly interesting in the context of the so-called hygiene hypothesis (12).

The aim of this study was to test *AMCase* gene variants for association with childhood bronchial asthma.

METHODS

Approval

The study was approved by the ethical committees of the University of Freiburg and Berlin, respectively. A statement of informed consent was signed by all participants or, in the case of children, signed by their parents.

Subjects

A total of 322 children with bronchial asthma (ages, 5–18 yr; mean age, 9.9 yr; 34% female, 66% male) were recruited between July 2000 and January 2005. An 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 any family history of allergic diseases.

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Recruitment criteria were as follows: a clear-cut history of asthmatic symptoms, the use of antiasthmatic medication, and the presence of bronchial hyperreactivity. Bronchial hyperreactivity was defined as a fall in FEV_1 by at least 15% in histamine testing or exercise provocation after standard lung function testing procedure (13). The protocol for clinical testing has been described in detail previously (14). Only children who fulfilled the criteria bronchial hyperreactivity were included.

Adult Control Population

A total of 270 randomly chosen probands were used as control subjects (ages, 19–40 yr; mean age, 24.6 yr; 53% female, 47% male). They originated from the same area of Germany. No medical history was taken and no medical testing was performed on control subjects.

Pediatric Control Population

These children were recruited as part of the Multicenter Allergy Study cohort that has been described in detail previously (15). Briefly, 1,314 children born in 1990 were enrolled in a prospective noninterventional birth cohort study. The infants were followed from birth to 10 yr of age. Clinical evaluations included structured interviews focusing on relevant symptoms of asthma, bronchial hyperreactivity, and atopy. DNA was collected from 888 children. Children who had a negative history for wheezing and no diagnosis of asthma ever were selected as pediatric control subjects (n = 565).

Genotyping

DNA was extracted from peripheral blood leukocytes after standard protocols (DNA Midikit; Qiagen, Germany). All polymorphisms were typed by means of restriction fragment length polymorphisms; *see* Table 1 for the conditions.

Sequencing

The promotor and all exons of *AMCase* were sequenced in 15 people with asthma and 15 control subjects. Furthermore, for each polymorphism under investigation, three control subjects (homozygous wild-type, heterozygous, and homozygous mutation) were sequenced by the dideoxy chain termination method (16) using the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems, Darmstadt, Germany). All of the following studies included these reference individuals.

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 (17) as implemented in the program Finetti (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl and http://ihg.gsf.de/linkage/download/finetti.zip). Hardy-Weinberg equilibrium was calculated for each polymorphism in the populations also using the Finetti program. In addition to analyses based on single polymorphisms, we performed haplotype frequency estimations by using the programs FASTEHPLUS (18) and FAMHAP (19), as previously described in detail (20).

RESULTS

Identification of Polymorphisms

We started our investigation by genotyping those three amino acid variants that were already listed in public single nucleotide polymorphism (SNP) databases (http://snpper.chip.org/ and http://www.ncbi.nlm.nih.gov/SNP/); that is, rs2275253 (= amino acid exchange I231V), rs2275254 (= F246S), and rs2256721 (= V324G). Association analysis of these three SNPs showed no association with bronchial asthma; however, haplotypes of these SNPs were in association with asthma with the p value of 0.0001.

Consecutively, we sequenced all 11 exons and the promotor region up to −1,000 bp before base 1 in exon 1 in 30 individuals. We identified the following polymorphisms in the promotor region (for those SNPs that were already listed in public databases the rs number is given): rs12023321, rs12033184, rs11102234, C/T 23 bp in front of rs12023459, rs12023459, and rs11102235. We found these variants (the given base pair number corresponds to the number in the respective exon) in exon 4, A47G (rs3818822); in exon 5 A50G, leading to the amino acid exchange K17R; and in exon 10, T95C, leading to the amino acid exchange F269S.

Genotyping

All six SNPs located in exons and one promotor SNP were typed on 322 patients with asthma and the two control populations. The allelic frequency and the Hardy-Weinberg equilibrium in all three populations are given in Table 2. All SNPs were in the Hardy-Weinberg equilibrium in control subjects and patients with asthma. The genotype distribution is given in Table 3.

Association Analyses

The results of the association analyses by the Armitage's trend test is given in Table 3. The newly identified amino acid variant K17R showed clear association with asthma using the adult and the pediatric control population (p = 0.0031 and p = 0.0172, respectively). In addition, the next nearby located noncoding SNP in exon 4 rs3818822 showed association with the disease (p = 0.0197 and p = 0.0003, respectively).

Haplotype Analyses in the Adult Control Population

All haplotypes with a frequency of at least 0.01 in either population are listed in Table 4. The distribution of the haplotypes differed significantly between patients with asthma and adult

TABLE 1. CONDITIONS FOR THE RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Polymorphism	Base Pair Substitution	Primer	PCR Conditions	Restriction Enzyme
rs12033184	A/C	5′-ACAGTACGCAGCTGAGCAAA-3′ 5′-TGAGTTTTGGGGGAACTCAT-5′	65°–55°C in −0.5°C steps, 40 cycles	MboII
rs3818822	A/G	5′-CCATTGGAGGCTGGAACTCC-3′ 5′-CCCCAAATGTCCACCAAAT-3′	51°C, 35 cycles	HpaII
K17R	A/G	5′-GTCTCACCCTGCCTTCTTTG-3′ 5′-ACCCAATTCTCCTCGGAAAG-3′	56°C, 35 cycles	ApoI
rs2275253	A/G	5′-CCTGAAAAATGGAGCCACTC-3′ 5′-GCCTTCTTCAGGGTGGAGAT-3′	59°C, 40 cycles	TaiI
rs2275254	C/T	5′-CCTGAAAAATGGAGCCACTC-3′ 5′-GCCTTCTTCAGGGTGGAGAT-3′	59°C, 40 cycles	ApoI
F269S	T/C	5′-CCTGAAAAATGGAGCCACTC-3′ 5′-GTGGGAAGACATCAGGGTTG-3′	60°C, 35 cycles	AluI
rs2256721	G/T	5′-AGGCAGTGGATTCTGTGCCG-3′ 5′-TAGGTAGGGGCAACATGTCC-3′	56°C, 40 cycles	NgoMIV

Definition of abbreviation: PCR = polymerase chain reaction.

TABLE 2. FREQUENCY OF THE POLYMORPHISMS AND RESULTS OF HARDY-WEINBERG EQUILIBRIUM FOR THE ASTHMA AND THE CONTROL POPULATION OBTAINED WITH THE PROGRAM FINETTI

			Allelic Frequency			Hardy-Weinberg Equilibrium		
				Control Subjects			Control Subjects	
Polymorphism	Position	Amino Acid Exchange	Asthma	Adults	Children	Asthma	Adults	Children
Rs12033184	Promotor	—	0.417	0.418	0.433	0.962	0.445	0.2782
Rs3818822	Exon 4	—	0.110	0.158	0.175	0.082	0.843	0.6749
	Exon 5	K17R	0.886	0.823	0.843	0.158	0.953	0.4782
Rs2275253	Exon 9	I231V	0.323	0.280	0.279	0.452	0.129	0.9234
Rs2275254	Exon 10	F246S	0.625	0.617	0.598	0.065	0.103	0.2117
	Exon 10	F269S	0.967	0.965	0.966	1.000	0.280	1.000
Rs2256721	Exon 11	V324G	0.696	0.734	0.733	0.125	0.119	0.1849

Given is the frequency of the "wild-type" allele according to the sequence NM_201653.

control subjects by p = 0.000020 (calculated with FAMHAP; 100.000 simulations). Using the program FASTEHPLUS, a χ^2 of 84.24 was obtained. Correcting for all theoretically possible haplotypes minus 1—that is, for 127 degrees of freedom (df)—a p value of 0.9987 was obtained. However, correcting only for those 40 haplotypes that actually occurred in our populations, the p value was 0.000039 (df, 39).

Haplotype Analyses in the Pediatric Control Population

The haplotype distribution of the pediatric population is also shown in Table 4. The haplotypes with the widest difference in frequency between control subjects and patients with asthma are shown in bold. It is obvious that the pediatric control population differs even more from the patients with asthma than the adult control subjects. This accounts particularly for the haplotypes 1 2 1 1 2 1 2 and 2 2 1 1 2 1 2. Interestingly, these two haplotypes only differ in the first polymorphism, rs12033184. This SNP does not show any association with asthma when taken alone, but thus seems to be very important in the context of haplotypes.

The high difference in haplotype distribution is reflected by a p value for association of 0.000000 gained by FAMHAP and a χ^2 of 114.08 by FASTEHPLUS. The latter leads to a p value of less than 10^{-10} when correcting for those haplotypes that occurred in the populations.

DISCUSSION

By sequencing *AMCase* in 30 individuals, we identified three known and two new amino acid variants. These polymorphisms and two SNPs of the 5′ region were genotyped on children with asthma, adult control subjects, and a pediatric control population. We found association of the newly identified variant K17R and the nearby noncoding polymorphism rs3818822 with asthma. In addition, haplotype analyses revealed very strong association of haplotypes with the disease (asthmatic population vs. pediatric control subjects, $p < 10^{-10}$).

We started our study by using randomly chosen adults as control subjects. No clinical testing was performed on them and so it is likely that about 10% of these control subjects have asthma. Including people with asthma in the control population enhances the risk to get false-negative results. However, the risk of getting a false-positive association is smaller than that if using control subjects that have no asthma. Therefore, the results with these control subjects should tend to underestimate rather than overestimate the observed association.

To further confirm the association, we then evaluated the polymorphisms in a second control population. This population consists of 565 children born in 1990 who had never wheezed and were never diagnosed of having asthma up to the age of 10 yr. Using these control subjects, the association became much stronger, especially in haplotype analysis.

In detail, the newly identified amino acid exchange lysine to arginine on position 17 showed association with asthma. It is located only seven amino acids in front of the catalytic center of AMCase (21). It is therefore plausible that this amino acid exchange may modulate the catalytic activity of AMCase. An arginine-to-lysine substitution may be considered conservative, but there are examples of substantial modulation of activity by this substitution in enzyme systems (22, 23). An artificially introduced mutation in the catalytic center destroys catalytic activity of chitotriosidase (24).

The SNP located closest to K17R—rs3818822—also showed association with asthma, and when pediatric control subjects

TABLE 3. GENOTYPE DISTRIBUTIONS (HOMOZYGOUS FOR WILD-TYPE, HETEROZYGOUS, HOMOZYGOUS FOR MUTATION) AND RESULTS OF THE ASSOCIATION ANALYSES WITH ARMITAGE'S TREND TEST FOR BOTH CONTROL POPULATIONS

				p Value Asthma	
Polymorphism	Asthma Genotypes	Adult Controls	Child Controls	Adults	Children
Rs12033184	55;155;108	44;137;88	99;289;175	0.9567	0.5122
Rs3818822	7;56;254	7;69;186	18;154;372	0.0197	0.0003
K17R	253;59;7	176;76;8	402;144;16	0.0031	0.0172
Rs2275253	30;144;142	26;98;144	43;226;291	0.1150	0.0493
Rs2275254	133;135;53	109;115;46	208;256;98	0.7890	0.2847
F269S	252;17;1	294;21;0	527;38;0	0.8631	0.9732
Rs2256721	150;148;24	150;95;24	297;233;34	0.144	0.0804

TABLE 4. RESULTS OF THE HAPLOTYPE FREQUENCY ESTIMATION OBTAINED BY FAMHAP

Haplotype	Asthma	Control Subjects	
		Adults	Children
1 1 1 2 2 1 1	0.023335	0.023489	0.017236
1 2 1 1 1 1 2	0.013974	0.000000	0.001096
1 2 1 1 2 1 1	0.000000	0.017351	0.001821
1 2 1 1 2 1 2	**0.105921**	**0.109709**	**0.166181**
1 2 1 2 1 1 1	**0.232742**	**0.194970**	**0.182000**
1 2 1 2 1 2 1	0.030304	0.022670	0.030234
1 2 2 2 1 1 1	0.000000	0.013705	0.010822
2 1 1 2 1 1 1	0.011039	0.018187	0.018564
2 1 1 2 2 1 1	**0.073852**	**0.085775**	**0.102604**
2 1 2 2 1 1 1	0.000000	0.010554	0.000000
2 2 1 1 1 1 1	0.013012	0.000000	0.004041
2 2 1 1 1 1 2	0.028990	0.011951	0.001066
2 2 1 1 2 1 2	**0.146976**	**0.102581**	**0.076349**
2 2 1 2 1 1 1	0.188650	0.173677	0.191984
2 2 1 2 2 1 1	0.013756	0.014569	0.011107
2 2 2 2 1 1 1	**0.099457**	**0.139256**	**0.142457**

Each number indicate the allele of one single nucleotide polymorphism (SNP): 1 = wild-type; 2 = mutation. The SNPs are listed in the order of their occurrence in the gene. Shown are haplotypes with a frequency $\geq$ 0.01 in at least one population. The haplotypes with the widest difference in frequency are shown in boldface type.

were used, significance was even higher than the one seen with K17R itself ($p = 0.0003$ vs. 0.0172). Because both SNPs are in very tight linkage equilibrium in both populations, this could still reflect a functional role of K17R on the disease.

The promoter polymorphism rs12033184 was not associated with the disease when analyzed alone. However, it seems to be important in the context of haplotypes, especially when comparing children with asthma with pediatric control subjects. Here again, functional analyses are needed to clarify which polymorphism is responsible for the effect.

Haplotypes analyses were performed by using the program FAMHAP, which revealed strong association of the haplotypes with asthma ($p = 0.000020$ and $p < 0.0000001$, respectively, for both control populations). FAMHAP permutes the disease status and reestimates haplotype frequencies for each simulated replicate. In our study, we performed 100,000 simulations with FAMHAP. These replications should lead to more accurate p values than obtained by methods that perform haplotype-frequency estimation only once, assign haplotypes to the individuals, and then permute these haplotypes (19).

Nevertheless, we sought to confirm the results by using a second program for haplotype analysis, FASTEHPLUS. FASTEHPLUS performs correction for all theoretically possible haplotypes by calculating the degree of freedom as numbers of haplotypes minus 1. In our study with seven SNPs, there are 128 haplotypes and thus the χ^2 is corrected for 127 df ($p = 0.9987$). Taking into account only those haplotypes that actually occurred in our asthmatic or adult control population, the p value is 0.000038 ($df = 39$). Using the pediatric control population, the corrected p value was less than 10^{-10}.

So far, only little is known about the function of chitinases in humans. Studies in mice imply that the enzymes are important in resistance to parasites containing chitin (25). It could be speculated that, in humans, higher chitinase activity might be connected to increased resistance to common pathogens. Chitinases have been highly conserved between different species, but human AMCase exhibits five amino acid polymorphisms (1), and their effect on AMCase activity and substrate specificity remains to be determined. However, because AMCase seems to be involved in the pathogenesis of asthma in mouse models, increased AMCase activity might favor the development of asthma. If this were indeed the case, it would add further support the so-called hygiene hypothesis. Functional studies are needed to clarify this issue, and replication of our results in additional populations is needed.

In conclusion, we report on a possible association of *AMCase* genetic variants with bronchial asthma. The involvement of AMCase in the pathophysiology of asthma highlights a previously poorly explored pathogenetic pathway that might be interesting for the development of new therapeutic agents (26, 27).

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